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POTENCY OF PURIFIED TUBERCULIN DETERMINED BY THE SHOCK METHOD ON HAMSTERS

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Jespersen, A. Potency of purified tuberculin determined by the shock method on hamsters. Acta path. microbiol. scand. Sect. C, 84 1–4 1976.

Two lots of purified protein derivative of tuberculin with different potencies determined by skin tests on guinea pigs, were examined for their capacity to induce shock in hamsters sensitized with BCG. Groups of hamsters were injected with varying doses of a highly potent strain of BCG grown in Dubos fluid medium. 4–5 weeks later two groups of animals sensitized with corresponding doses were injected intraperitoneally with 1 mg of each lot of tuberculin. 12 of the 20 hamsters given the strong tuberculin died, while all those given the weak tuberculin survived. An attempt was made to evaluate the mutual potency of the two lots of tuberculin by injecting groups of sensitized animals with 1 mg strong or 4 mg weak tuberculin. The difference in the number of deaths from shock in the two groups was not significant.

Key words: Purified tuberculin; potency; shock method; hamsters.

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Previous experiments (*Jespersen* 1973) have shown that the dose of purified tuberculin which can induce shock in BCG immunized hamsters is about the same as that required for similarly immunized guinea pigs. Furthermore, it has been proved that the potency of a BCG vaccine can be determined on the basis of the tuberculin shock method. The aim of the present study was to examine whether the shock method in hamsters sensitized with a strongly potent strain of BCG can be used to determine the potency of a tuberculin.

MATERIAL AND METHODS

Experimental animals. The 2 to 3 month old hamsters were bred at Statens Seruminstitut farm. The female and male animals were placed separately at random in guinea pig cages partitioned into two sections. Each experimental group consisted of equal number of females and males.

BCG strain. The strain is a laboratory strain from the Dubos Laboratory supplied from Copenhagen in 1951 and returned to Copenhagen in April 1960 (designated "Dubos Copenhagen"). In several experiments it has been shown that this strain has a strong lethal capacity for hamsters (*Jespersen & Bentzon* 1964a, b, 1967). A Löwenstein-Jensen culture from a spontaneously dead hamster injected with the strain in the spring of 1974 was inoculated into Dubos fluid medium and the culture used when 12 days old. Inoculation of suitable dilutions on Löwenstein-Jensen medium showed that 1 ml of the undiluted bacterial suspension contained 33 × 10 viable units. In the

TABLE 1 *Survival Times in Days of Hamsters Sensitized with 33 × 10⁵ or 33 × 10⁶ Viable Units BCG and Challenged with 1 mg Lot 42 or Lot 62 RT 23 4 to 5 Weeks Later*

33 × 10⁵ viable units BCG		33 × 10⁶ viable units BCG	
62/RT 23 1 mg	42/RT 23 1 mg	62/RT 23 1 mg	42/RT 23 1 mg
Survivor	1 F	Survivor	1 M
Survivor	1 F	Survivor	1 M
Survivor	2 M	Survivor	1 M
Survivor	2 F	Survivor	2 M
Survivor	3 F	Survivor	4 F
Survivor	Survivor	Survivor	7 M
Survivor	Survivor	Survivor	7 F
Survivor	Survivor	Survivor	Survivor
Survivor	Survivor	Survivor	Survivor
Survivor	Survivor	*	Survivor

* Died in the interval between vaccination and injection of tuberculin.
F = female.
M = male.

experiment where the animals were resensitized the suspension used for the second injection contained 18 × 10⁶ viable units.

Tuberculin RT 23 is a large batch of purified tuberculin (more than 500 g) comprising a mixture of 77 smaller lots produced at the Tuberculin Department, Statens Seruminstitut, Copenhagen, during the years 1954–1957 A description of the preparation methods and the biological assays performed has been published previously (*Magnusson & Bentzon* 1958 *Guld et al* 1958) Skin testing of BCG vaccinated guinea pigs showed that lots 29 and 42 had significantly higher potency and lots 62 and 66 significantly lower potency than the average for all lots. A corresponding study carried out in 1973 (*Nilsson & Magnusson*) revealed that the potency relationship of lots 42 and 62 to RT 23 was 1 82 and 0 44 respectively *i.e* a mutual relationship of 4 1 These two lots were examined in the present study Samples stored in powder form at 20–25 C were dissolved in 0 15 M phosphate buffered sodium chloride solution pH 7 4 at a concentration of 1 mg/ml*

Experimental Three groups of hamsters, each consisting of 20 animals, were injected intraperitoneally with the following doses of BCG 1 ml undiluted culture (10⁰) 1 ml of a culture diluted 10 times (10⁻¹) and 1 ml of a culture diluted 100 times (10⁻²) 4 to 5 weeks later half of the animals in the 10⁰ and 10⁻¹ groups were injected intraperitoneally with 1 mg tuberculin lot 42 and the other half with 1 mg of lot 62

* The writer is grateful to *Mogens Magnusson* Chief of the Tuberculin Department, for the preparation and provision of the tuberculin.

Judged by the number of deaths from shock, it could not be expected that any of the group injected with 10⁻² BCG would die of shock. A second injection of BCG was therefore given in an attempt to increase the shock sensitivity 48 days after the first injection the animals were injected intraperitoneally with 1 ml undiluted culture. The tuberculin was given intraperitoneally 14 days later In order to obtain an evaluation of the potency relationship between the two lots of tuberculin, one half of the animals were injected with 1 mg of the strong and the other half with 4 mg of the weak tuberculin These doses were selected on the basis of the above-mentioned potency determination on guinea pigs (*Nilsson & Magnusson* 1973)

RESULTS

Experiment 1 (Table 1) The strong tuberculin induced shock in seven out of ten hamsters sensitized with undiluted BCG culture, and in five out of ten animals sensitized with a culture diluted 10 times. There is no significant difference between the two groups of animals, either from the point of view of the number of survivors or the length of the survival times. The weak tuberculin was not able to induce shock in any of the 19 sensitized animals.

Experiment 2 (Table 2) Shock was induced in nine out of ten animals by injection of 1 mg of the strong tuberculin and in

six out of ten animals by injection of 4 mg of the weak tuberculin. The difference between the groups of animals is not significant, either as regards the number of survivors or the length of the survival times. Comparison of the group of animals in Experiment 1 sensitized with undiluted BCG culture and the corresponding resensitized group in Experiment 2 shows that the number of survivors was 3 and 1 respectively and that the survival times were shorter in the resensitized group. However, the difference is not significant.

TABLE 2. *Survival Times in Days of Hamsters Sensitized with 33 × 10⁴ Viable Units BCG, Resensitized with 18 × 10⁴ Viable Units 48 Days Later and Challenged with 1 mg Lot 42 or 4 mg Lot 62 RT 23 2 Weeks Later*

62/RT 23 4 mg	42/RT 23 1 mg
1 F	1 F
1 M	1 M
1 F	1 F
2 M	1 M
2 M	1 F
3 F	1 M
Survivor	1 F
Survivor	2 M
Survivor	2 M
Survivor	Survivor

F female
M male

DISCUSSION

The tuberculins compared in the present study were two of the 77 lots which comprise batch RT 23. After their preparation, all lots were tested by means of skin tests on BCG sensitized guinea pigs (*Magnusson & Bentzon* 1958, *Mauld* 1970). Lots 29 and 42 were found to be significantly stronger and lots 62 and 66 significantly weaker than the average for all lots. The four lots have also been tested on human beings in Norway (*Galtung & Waaler* 1970). Using the Pirquet test, it was found that equal portions of lots 29 and 42 had a six to seven times stronger effect than equal portions of lots 62 and 66. The mixture of lots 29 and 42 had a stronger effect than RT 23, while the mixture of lots 62 and 66 was weaker.

The four lots have also been compared in in vitro experiments (*Nilsson & Magnusson* 1973). The potency of the tuberculins was determined by their capacity to induce synthesis of deoxyribonucleic acid in cultures of lymphocytes from human subjects vaccinated with BCG. Comparison of the effect of the four lots with that of RT 23 on the uptake of (¹⁴C) thymidine by the lymphocytes showed that also by this method lots 29 and 42 were significantly stronger than lots 62 and 66. As mentioned previously, it was found by skin testing of BCG vaccinated guinea pigs that the mutual potency relationship for lots 42 and 62 was 4:1.

The shock experiments on hamsters revealed a clear difference in potency between lots 42 and 62, and the results are compatible with the fact that lot 42 is about four times as strong as lot 62. The amount of the two lots of tuberculin available did not permit the performance of a comprehensive relative potency determination, which presupposes the use of at least two suitable doses of each of the preparations examined.

The method could certainly be improved by the adaptation of the following mutually interacting factors:

1) *Bacterial doses.* The present experiment indicates that two doses of BCG give stronger sensitization than one single dose. The same has been shown in experiments on mice (*Dietrich et al.* 1974). An even stronger sensitization of hamsters could probably be achieved by using two doses of a virulent bacterial strain.

2) *Interval between injection of bacteria and challenge with tuberculin.* The interval of 4 to 5 weeks in the present study is probably too long if a large dose of BCG is used for sensitization. In *Han & Waser's* (1967) experiments on mice, the maximum tuberculin sensitivity was obtained as early as after 2 to 3 weeks, after which it declined slowly during the following months. *Dietrich et al.* (1974), who immunized mice with two doses of BCG, found that the maximum effect was

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33 × 10³ viable units BCG		33 × 10⁴ viable units BCG	
62/RT 23 1 mg	42/RT 23 1 mg	62/RT 23 1 mg	42/RT 23 1 mg
Survivor	1 F	Survivor	1 M
Survivor	1 F	Survivor	1 M
Survivor	2 M	Survivor	1 M
Survivor	2 F	Survivor	2 M
Survivor	3 F	Survivor	4 F
Survivor	Survivor	Survivor	7 M
Survivor	Survivor	Survivor	7 F
Survivor	Survivor	Survivor	Survivor
Survivor	Survivor	Survivor	Survivor
Survivor	Survivor	*	Survivor

* Died in the interval between vaccination and injection of tuberculin.
F = female
M = male.

experiment where the animals were resensitized, the suspension used for the second injection contained 18 × 10⁴ viable units.

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Experimental Three groups of hamsters, each consisting of 20 animals, were injected intraperitoneally with the following doses of BCG 1 ml undiluted culture (10⁴) 1 ml of a culture diluted 10 times (10⁻¹) and 1 ml of a culture diluted 100 times (10⁻²) 4 to 5 weeks later half of the animals in the 10⁰ and 10⁻¹ groups were injected intraperitoneally with 1 mg tuberculin lot 42 and the other half with 1 mg of lot 62

* The writer is grateful to *Mogens Magnusson* Chief of the Tuberculin Department for the preparation and provision of the tuberculin.

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Experiment 2 (Table 2) Shock was induced in nine out of ten animals by injection of 1 mg of the strong tuberculin and in

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THE EFFECT OF STEROIDS ON ADHESIVENESS ROSETTE FORMING ABILITY AND SURVIVAL OF CULTURED HUMAN MONONUCLEAR CELLS

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Viken, K. E. The effect of steroids on adhesiveness, rosette-forming ability and survival of cultured, human mononuclear cells. Acta path. microbiol. scand. Sect. C, 84 5–12, 1976.

The effect of different steroids on human mononuclear blood cells during the first 90 minutes of culture *in vitro* was tested. Cortisol and testosterone were found to increase the ability of lymphocytes to adhere on plastic surfaces. None of the other steroids tested exhibited this effect. Cortisol and corticosterone were found to reduce the number of surviving macrophages in the culture dishes, tested 4 to 8 days after the exposure to the drugs. No lysis of mononuclear cells could be detected following addition of cortisol in doses up to 10⁻ mg/ml. The findings support the previous statements that human lymphocytes are more cortisol-resistant than those of mouse rat and rabbit.

Key words Mononuclear blood cells/human effect of steroids.

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Received 30 73 Accepted 17 III.75

Cortisol (hydrocortisone) has been used for many years in medicine as an anti-inflammatory and immunosuppressive drug. Its mode of action on the cellular level is still not understood. *In vivo* the most predominant finding after cortisol administration is a profound reduction of lymphocytes in the peripheral circulation (9) a transient fall in the number of circulating monocytes (5 7) and a reduced number of macrophages (8) and granulocytes (1) at the site of inflammation. Results of experiments with rat and mouse lymphocytes indicated lymphocytolysis as the explanation of the fall in number of circulating lymphocytes. Later studies, however show that lymphocytes from man and guinea pigs are far more resistant to cortisol and are referred to as "cortisolresistant" (3, 8). The fall in number of lymphocytes in the circulation is too rapid to be caused by inhibition of the production of these cells. The lymphocytes must then leave the circulation either by an increased diapedesis or be sequestered.

In the study to be presented, human mononuclear blood cells were tested for possible effect of cortisol and other steroids on cellular adhesiveness and on the survival of monocytes after a 90 minutes exposure to the steroids at culture start. The non-attached cells were tested for possible changes in the proportion

achieved about 10 days after the second injection and that the sensitivity had disappeared almost completely 40 days later

3) *Dose of tuberculin* The size of the lethal tuberculin dose is dependent on the dose of bacteria injected and on the interval between injection of bacteria and challenge It will be seen from the experiments that 1 mg tuberculin would be a suitable starting point for selection of the doses for potency determination irrespective of whether one or two injections of BCG are used for sensitization If sensitization is performed with a virulent strain the dose of tuberculin can probably be reduced

The above mentioned studies of strong and weak lots of RT 23 indicate that there is a positive correlation between the results of skin tests on tuberculous guinea pigs and tuberculous human beings and the results of the shock test on sensitized hamsters. This applies to various batches of purified tuberculin for which the same preparation methods have been used With tuberculins prepared by different methods, the potency relationship may be dependent on whether it is determined by skin tests or by the shock method on guinea pigs (*Lind* 1948) It would therefore be of interest to investigate what the result of the shock method on hamsters would have been were such preparations used

REFERENCES

Dietrich F W Gisler R H Percin C & Schumann G Tuberculin shock in mice Relationship between systemic reactions to tuberculin and endotoxin and lack of correlation between tuberculin shock and cellular immunity to tuberculin (38024) Proc. Soc. exp. Biol. (N Y) *145* 1409–1417 1974

Galtung O & Waaler H Personal communication

Guld J Bentzon M W Bleiker M A., Griep W A Magnusson M & Waaler H Standardization of a new batch of purified tuberculin (PPD) intended for international use Bull Wld Hlth Org *19* 845–951 1958.

Han S H & Weiser R S Systemic tuberculin sensitivity in mice. I Factors contributing to active tuberculin shock. J Immunol *98* 1152 1157 1967

Jespersen A & Bentzon M W The virulence of various strains of BCG determined on the golden hamster Acta tuberc. scand *44* 222 249 1964*a*.

Jespersen A & Bentzon M W The acquired resistance to tuberculosis induced by BCG vaccine assayed by a quantitative method on red mice (*Clethrionomys g glareolus Schreb*) 2. Vaccination effect of BCG strains strongly or moderately virulent for hamsters. Acta tuberc scand *44* 276 289 1964*b*

Jespersen A & Bentzon M W Relationship between tuberculin sensitivity and acquired resistance in guinea pigs vaccinated with BCG strains of different virulence Acta path. microbiol scand *71* 114–131 1967

Jespersen A The potency of BCG vaccine determined by the tuberculin shock method on hamsters. Acta path. microbiol scand Sect. B, *81* 337–341 1973

Lind P Purified tuberculin its preparation and properties. Acta tuberc. scand. *22* 287–432, 1948.

Magnusson M & Bentzon M W Preparation of purified tuberculin RT 23 Bull Wld Hlth Org *19* 829 843 1958

Mavild J Personal communication

Nilsson B S & Magnusson M Comparison of the biologic activity of tuberculins by the use of lymphocyte cultures Amer Rev resp Dis *108* 565 570 1973

TABLE 1 *The Effect of Cortisol on Different Functions of Mononuclear Cells During the First 90 Minutes of Culture*

Drug conc. mg/ml	Cellular adhesiveness	Rosette-forming ability of lymphocytes	Survival
10^{-}	165 ± 64	120 ± 44	81 ± 30
10	157 ± 64	119 ± 41	77 ± 18
10^{-}[illegible]	159 ± 53	140 ± 81	95 ± 24
10^{-}	158 ± 63	124 ± 67	98 ± 15
10^{-}[illegible]	180 ± 66	121 ± 46	104 ± 11
0*	100*	100*	100*

The figures listed are the means ± SD of the results from 12 experiments, carried out as described as test procedure 1. The results are expressed as per cent of control values. The experiments were performed in duplicate petri dishes.

Control without drug addition: Cellular adhesiveness 1.15 ± 0.70, range 0.13–2.38 × 10[illegible] adhesive cells per culture dish. Rosette-forming ability 21.0 ± 6.0, *range 7.0–30.6 per cent rosette-forming* lymphocytes of the total number of non-adhesive cells. Survival 134 ± 88, range 50–275 × 10[illegible] cells per culture dish after 8 days of culturing.

(17β-hydroxyandrost-4-en-3-one), desoxycorticosterone (4-pregnen-21-OL 3,20-dione), tetrahydrocortisol (*3β-pregnan-3α, 11β, 17α, 21-tetrol-20 one*) and corticosterone (4-pregnen-11β, 21-diol-3,20 dione) were obtained from Steraloids, Inc., USA. Cortisol was added directly to the culture dishes after being dissolved in sterile, distilled water. The other steroids were dissolved *in medium 199 with* 20 per cent human A serum and membrane filter sterilized (Millipore, 0.22 μ) before use.

Test Procedure 1 (10)

2.3 ml of the cell suspension was added to each plastic petri dish, diameter 5 cm (Nunclon, Denmark), together with steroids in concentrations from 10 to 10 mg/ml. The culture dishes were incubated at 37 °C in a National CO_2-incubator with 5 *per cent* CO_2 in air and 100 per cent humidity. After 90 minutes' incubation, the non-adhesive cells were harvested, the cell layer was washed once in Hanks' BSS and fresh complete medium was added to the culture dishes.

a) *Cellular adhesiveness.* The number of adhesive cells after 90 minutes' incubation was estimated by counting the non-attached cells in an electronic particle counter (Coulter type Fn). The figures given are the differences between the numbers of cells added and the numbers of non-adhesive cells.

b) *Rosette-forming ability of lymphocytes.* The test was performed on the non-adhesive cells as described in *detail by Odegaard* (10), slightly modified according to *M... et al* (4). The values given are the percentages of rosette forming lymphocytes with more than 5 sheep erythrocytes attached to the cell surfaces, compared with the total number of lymphocytes.

c) *Survival.* The survival of the adhesive cells was quantified by counting adhesive cells in an inverted phase contrast microscope (Reichert). The figures given are the estimated numbers of cells per petri dish after counting 20 free selected microscope fields, expressed as percentages of the numbers of cells in the controls.

Statistics

The *experiments* were performed in duplicate or triplicate culture dishes. The mean values from each concentration were calculated, and the results from each experiments were expressed as percentage of control values. The figures presented are the mean values *from 6–12* experiments ± standard deviation (SD). The p-values were calculated using the Wilcoxon Two-Sample test.

RESULTS

Cortisol

Cortisol was found to increase the number of adhesive cells, about 50 per cent being the mean ($p < 0.01$). The effect did not seem to be dose-dependent within the concentrations tested (Fig. 1). The magnitude of this effect, however, showed great variations from experiment to experiment, as expressed by the standard deviations (Table 1).

The effect on the rosette forming ability of lymphocytes was more uncertain. The mean values of the effect of cortisol on the rosette forming ability indicate that a larger number

of T and B-cells, using the rosette formation test as a T-cell indicator A possible lytic effect of cortisol was tested on radiolabelled mononuclear cells.

MATERIALS AND METHODS

Cell Separation

Mononuclear cells were separated from defibrinated venous blood from healthy donors as described by *Bøyum* (2) After being washed twice in Hanks balanced salt solution (BSS) the cells were suspended in medium 199 (Flow G B) supplemented with 20 per cent human A-serum and 50 µg Garamycin (Schering USA) per ml. The cell concentration was adjusted to 3×10^6 per ml. Contamination of polymorphonuclear cells has been shown to be approximately 5 per cent (11)

^{51}Cr-labelling of Mononuclear Cells

After being washed once in Hanks' BSS the cells were suspended in Tris-HCl/Hanks BSS and 10 µCi carrier free ^{51}Cr (Kjeller Norway) were added per ml The cells were incubated at 37 C for 30 minutes washed twice in Hanks BSS before being suspended in the culture medium (6)

Steroids

Cortisol Actocortin, (P C.F., Denmark) a soluble complex of cortisol was used. Testosterone

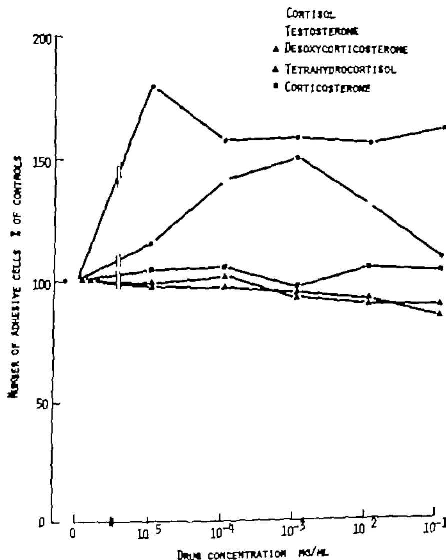


Fig 1 A comparison between the effects of different steroids on the adherence of mononuclear cells. The values plotted are the means of the results from 12 experiments using cortisol and 6 experiments using the other steroids expressed as per cent of controls.

TABLE 1 *The Effect of Cortisol on Different Functions of Mononuclear Cells D ring the First 90 Minutes of Culture*

Drug conc. µg/ml	Cellular adhesiveness	Rosette-forming bility of lymphocytes	Survival
10⁻	163 ± 64	120 ± 44	61 ± 30
10⁻	157 ± 64	119 ± 41	77 ± 18
10	159 ± 53	140 ± 81	95 ± 24
10	158 ± 65	124 ± 67	98 ± 13
10	180 ± 66	121 ± 46	104 ± 11
0*	100*	100*	100*

The figures listed are the means ± SD of the results from 12 experiments, carried out as described as test procedure I. The results are expressed as per cent of control values. The experiments were performed in duplicate petri dishes.

Control without drug addition. Cellular adhesiveness 1.15 ± 0.70 range 0.15–2.38 × 10⁶ adhesive cells per culture dish. Rosette-forming ability 21.0 ± 6.0, range 7.0–30.6 per cent rosette-forming lymphocytes of the total number of non-adhesive cells. Survival: 134 ± 68 range 50–275 × 10³ cells per culture dish after 8 days of culturing.

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Test Procedure I (10)

2.5 ml of the cell suspension was added to each plastic petri dish, diameter 5 cm (Nunclon, Denmark) together with steroids in concentrations from 10⁻³ to 10 µg/ml. The culture dishes were incubated at 37° C in a National CO_2-incubator with 5 per cent CO_2 in air and 100 per cent humidity. After 90 minutes' incubation, the non-adherent cells were harvested, the cell layer was washed once in Hanks BSS and fresh complete medium was added to the culture dishes.

a) *Cellular adhesiveness*. The number of adherent cells after 90 minutes incubation was estimated by counting the non-attached cells in an electronic particle counter (Coulter type F). The figures given are the differences between the numbers of cells added and the numbers of non-adherent cells.

b) *Rosette-forming ability of lymphocytes*. This test was performed on the non-adhesive cells as described in detail by *Odegaard* (10) slightly modified according to *Moore et al.* (4). The values given are the percentages of rosette-forming lymphocytes with more than 3 sheep erythrocytes attached to the cell surfaces, compared with the total number of lymphocytes.

c) *Survival*. The survival of the adhesive cells was quantified by counting adhesive cells in an inverted phase contrast microscope (Reichert). The figures given are the estimated numbers of cells per petri dish after counting 20 free selected microscopic fields, expressed as percentages of the numbers of cells in the controls.

Statistics

The experiments were performed in duplicate or triplicate culture dishes. The mean values from each concentration were calculated, and the results from each experiments were expressed as percentage of control values. The figures presented are the mean values from 6–12 experiments ± standard deviation (SD). The p-values were calculated using the Wilcoxon Two-Sample test.

RESULTS

Cortisol

Cortisol was found to increase the number of adhesive cells, about 50 per cent being the mean ($p < 0.01$). The effect did not seem to be dose-dependent within the concentrations tested (Fig. 1). The magnitude of this effect, however showed great variations from experiment to experiment, as expressed by the standard deviations (Table 1).

The effect on the rosette-forming ability of lymphocytes was more uncertain. The mean values of the effect of cortisol on the rosette forming ability indicate that a larger number

of T and B-cells, using the rosette-formation test as a T-cell indicator. A possible lytic effect of cortisol was tested on radiolabelled mononuclear cells.

MATERIALS AND METHODS

Cell Separation

Mononuclear cells were separated from defibrinated venous blood from healthy donors as described by *Bøyum* (2). After being washed twice in Hanks balanced salt solution (BSS) the cells were suspended in medium 199 (Flow G.B.) supplemented with 20 per cent human A-serum and 50 µg Garamycin (Schering USA) per ml. The cell concentration was adjusted to 3×10^6 per ml. Contamination of polymorphonuclear cells has been shown to be approximately 5 per cent (11).

^{51}Cr-labelling of Mononuclear Cells

After being washed once in Hanks BSS the cells were suspended in Tris-HCl/Hanks' BSS and 10 µCi carrier free ^{51}Cr (Kjeller Norway) were added per ml. The cells were incubated at 37° C for 30 minutes, washed twice in Hanks BSS before being suspended in the culture medium (6).

Steroids

Cortisol: Actocortin, (F.C.F., Denmark) a soluble complex of cortisol was used. Testosterone

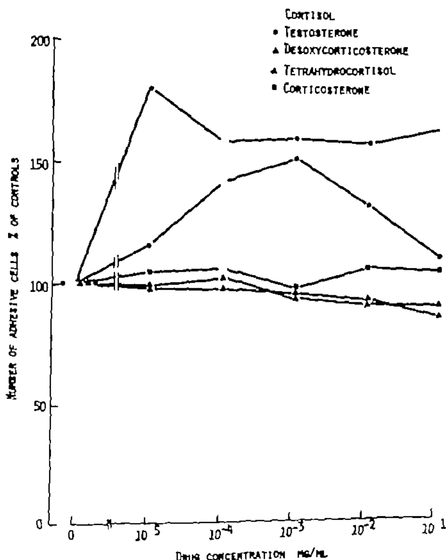


Fig. 1. A comparison between the effects of different steroids on the adherenceness of mononuclear cell. The values plotted are the means of the results from 12 experiments using cortisol and 6 experiments using the other steroids, expressed as per cent of controls.

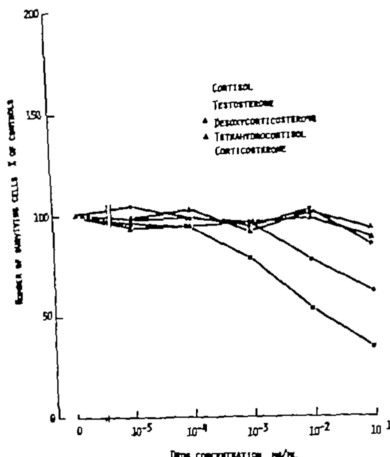


Fig. 3. The effect of different steroids on the survival of monocytes after 8 days of culture. For explanation of figures see Fig. 1.

TABLE 2. *The Effect of Testosterone on Different Functions of Mononuclear Cells During the First 90 Minutes of Culture*

Drug conc mg/ml	Cellular adhesiveness	Rosette-forming ability of lymphocytes	Survival
10	110 ± 23	92 ± 15	84 ± 20
10	132 ± 31	103 ± 16	101 ± 5
10	151 ± 34	90 ± 17	94 ± 10
10	142 ± 24	75 ± 20	98 ± 9
10	116 ± 17	84 ± 16	97 ± 7
0*	100*	100*	100*

The figures listed are the means ± SD of the results from 6 experiments, carried out as described in test procedure 1. The results are expressed as in Table 1.

Control without drug addition: Cellular adhesiveness 1.70 ± 0.55, range 1.18–2.33 × 10* adhesive cells per culture dish. Rosette-forming ability 31.0 ± 8.0, range 17.3–39.3 per cent rosette-forming lymphocytes of the total number of non-adherent cells. Survival 183 ± 61, range 104–260 × 10 cells per culture dish after 8 days of culturing.

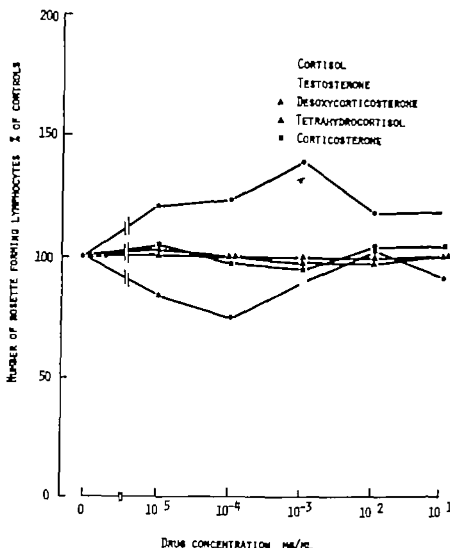


Fig 2 The effect of different steroids on the rosette forming ability of lymphocytes. For explanation of figures see Fig 1

of lymphocytes formed rosettes with sheep erythrocytes after exposure to cortisol or that a larger number of B-lymphocytes attached to the plastic surface (Fig 2) This effect however was not very marked (Table 1) ($p<0.05$ for the 10 ³ concentration)

The survival of monocytes was tested by counting viable (adhesive) cells 8 days after the exposure to the drug Cortisol in the concentration of 10 mg/ml was found to reduce the number of cells left in the petri dishes to about 60 per cent of the numbers in control cultures (Table 1 Fig 3) ($p<0.01$)

The effect of cortisol on ⁵¹Cr labelled mononuclear cells was also tested No significant effect was found in these experiments, indicating that there was no lysis of monocytes and lymphocytes during a short term exposure to cortisol (Fig 4)

Testosterone

The effect of testosterone on cellular adhesiveness was different from that of cortisol The number of adhesive cells was found to increase to a maximum of 50 per cent above the control values at the concentration of 10^{-3} mg/ml (Table 2 Fig 1) In the highest concentration used (10 mg/ml) the adhesiveness of mononuclear cells was found to be at the same level as in the control cultures.

In contrast to cortisol testosterone was

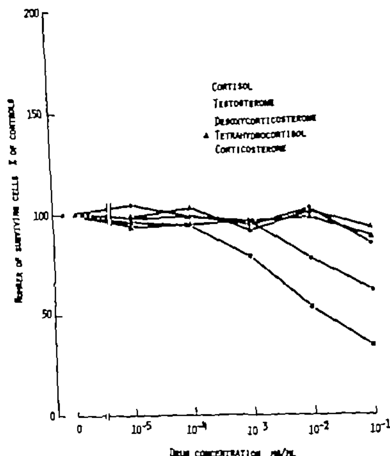


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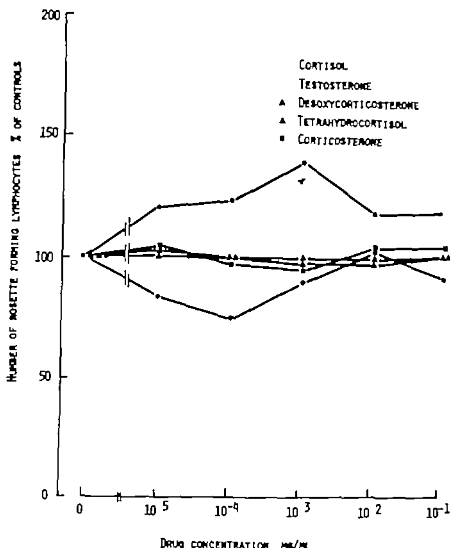


Fig 2 The effect of different steroids on the rosette-forming ability of lymphocytes. For explanation of figures see Fig 1

of lymphocytes formed rosettes with sheep erythrocytes after exposure to cortisol or that a larger number of B-lymphocytes attached to the plastic surface (Fig 2) This effect however was not very marked (Table 1) ($p<0.05$ for the 10^{3} concentration)

The survival of monocytes was tested by counting viable (adhesive) cells 8 days after the exposure to the drug Cortisol in the concentration of 10^{1} mg/ml was found to reduce the number of cells left in the petri dishes to about 60 per cent of the numbers in control cultures (Table 1 Fig 3) ($p<0.01$)

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DISCUSSION

The most predominant finding in this study was the increased attachment of mononuclear cells to a plastic surface if cortisol and testosterone were present in the medium. None of the other steroids tested exhibited this effect. Since all monocytes spread on plastic surface under the culture condition used, the results are likely to reflect an increased attachment of lymphocytes.

The percentage of rosette-forming lymphocytes with sheep red cells (T-cells) in the supernatants containing the non-adherent cells was not found to be influenced by treatment with the steroids tested in this system, except for a small increase in the number of rosette-forming lymphocytes in the non-adhesive cell fraction after treatment with cortisol. This finding may indicate a greater removal of adhering B-cells during cortisol treatment. However further investigations using a B-cell indicator is necessary if this finding is to be proved.

If the adhesive cells were cultured further in steroid-free environment, many attached cells were found to detach. After 4–8 days of culturing, the number of attached cells was not found to be affected by the steroid treatment, except for cortisol and corticosterone in the highest concentrations where a reduced number of cells were recorded.

It thus seems that cortisol is responsible for a transient alteration in the lymphocytes, probably by way of a membrane effect, making them more sticky to plastic surface, while no others of the steroids tested show this effect. In order to escape from the circulation and migrate into the tissues, the lymphocytes must attach themselves to the endothelial wall. Our results may indirectly support the theory that lymphocytes leave the circulation as an explanation for the rapid fall in circulating lymphocytes under cortisol treatment. The *in vitro* effect on adhesiveness shown in the experiments may be equivalent to a similar *in vivo* effect, by which the lymphocytes are made more sticky and attach to the endothelial wall.

In view of the biochemical structure of the different steroids tested, the increased attachment of the cells after treatment with cortisol and testosterone seems most likely to depend on a OH group in the 17 position a ketone in the 3 position or a double-bound between carbons 4 and 5 in the steroid molecule.

The reduced survival registered 8 days after the short term treatment with cortisol and corticosterone, is probably a result of either a toxic effect or a depression of the cell protein metabolism of these potent antimetabolic drugs.

Cortisol in doses from 10 to 10 mg/ml was not found to increase the release of ^{51}Cr from labelled mononuclear cell, indicating that there is no lysis of the cells. This supports the findings by *Claman et al* (3) according to whom human mononuclear cells are more resistant to cortisol-induced lysis than those of mouse rat and rabbit.

Technical assistance from Mrs. *B. H. Hansen* is gratefully acknowledged. I am also very grateful to Prof *J Lam ik* and Prof. *K. B. Elk* for critical discussions and help during the course of this study and the preparation of the manuscript.

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REFERENCES

1 *Boggs D R., Athens J W & Cartwright G E.* The effect of adrenal glucocorticosteroids upon the cellular composition of inflammatory exudates. Am. J Pathol *44* 763–775 1964

2. *Boyum A.* Separation of leucocytes from blood and bone marrow. Scand. J Clin. Lab. Invest. *21* Suppl 97 1968.

3 *Claman H N Moorhead J W & Benner W H.* Corticosteroids and lymphoid cells in vitro. 1 Hydrocortisone lysis of human, guinea-pig and mouse thymus cells. J Lab. Clin. Med. *78* 499–507 1971

4 *Moore A Benack M Mandel L., Cameron, J S Ellis F G., Boulton Jones M & Ogg C S* Clinical evaluation of rosette inhibition test in renal allotransplantation. Br Med. J *3* 271 275 1971

5 *Ruskert J S S… A L., Balcerzak S P., Ackerman G A & LoBuglio A F* Effects

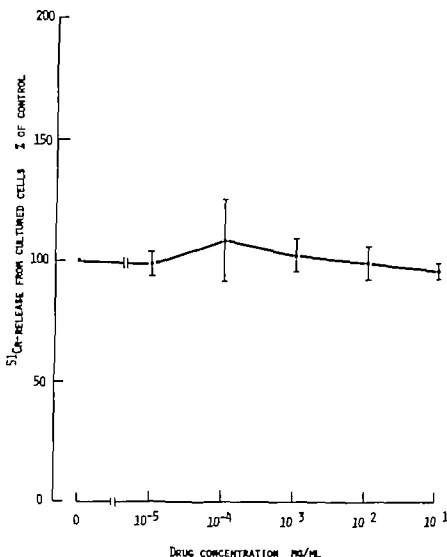


Fig 4 The effect of cortisol on ^{51}Cr release from mononuclear cells. The labelled cells were exposed to cortisol during the first 90 minutes of culture. The values given are the means ± SD of the results from 4 experiments, expressed as per cent of controls.

found to decrease slightly the number of rosette-forming lymphocytes in concentrations of 10^{-8}–10^{-4} mg/ml (Table 2 Fig 2) In the other concentrations uesd, no significant ef fect was discovered.

Testosterone was not found to have any significant effect on cell survival (Table 2 Fig 3)

Desoxycorticosterone and Tetrahydrocortisol

These steroids were found to have no ef fect either on the cell adhesiveness on the rosette-forming ability of lymphocytes or on the cell survival in the concentrations used and under the experimental conditions described here (Fig 1 2 3)

Corti osterone

Corticosterone exhibited no effect on the cellular adhesiveness and the rosette forming ability of lymphocytes in the concentrations used. The survival of macrophages cultured on coverslips for 8 days after exposure to the drug in concentrations of 10^{-2} and 10^{-1} mg/ml was found to be depressed (Fig 3)

Acta path. microbiol. scand. Sect. C, 84 13–22, 1976

THE EFFECT OF STEROIDS ON DIFFERENTIATION AND FUNCTION OF CULTURED, MONONUCLEAR CELLS

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Viken, K. E. The effect of steroids on differentiation and function of cultured, mononuclear cells. Acta path. microbiol. scand. Sect. C, 84 13–22 1976.

When monocytes had differentiated to macrophages during 8 days of culture *in vitro* without being exposed to steroids, neither the engulfement nor the digestion step of phagocytosis was found to be influenced by cortisol or the other steroids tested. This indicates that cortisol has no direct effect on the phagocytic function of macrophages in doses below 10^{-} mg/ml. Continuous exposure to cortisol during the period of differentiation resulted in a dose dependent inhibition of the differentiation of monocytes to macrophages. Testosterone, deoxy corticosterone and tetrahydrocortisol in concentration of 10^{-1} mg/ml, tested in the same way were found to be toxic to the cells. In lower concentrations, however these steroids were found to have no effect on the cultured cells. The impaired differentiation of monocytes is suggested as an additional explanation of the reduced number of macrophages appearing at the site of inflammation during cortisol treatment.

Key words Macrophages/human effect of steroids in vitro

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Received 30 75 Accepted 17 ii.75

As regards the effect of glucocorticosteroids on macrophages the most prominant in vivo finding is a reduced number of these cells at the site of inflammation during cortisol treatment (18) A reduction in the number of circulating monocytes which are precursor cells to the macrophages, is also observed during cortisol treatment (9 10) These findings indicate an impaired function of the mononuclear phagocytes and may in part explain the increased frequency of granulomatous and other infections (1 2, 5) the impaired hypersensitivity reaction (5) and other events concerning macrophages during exposure to high doses of cortisol. The mechanism by which cortisol inhibits the presence of macrophages at the site of inflammation however is still unknown. Neither is there any satisfactory explanation of the monocytopenia following cortisol administration (12) This report deals with the effect of cortisol and other steroids on the mononuclear phagocytes which serve both as immunological and scavenger cells in the defence system. The results obtained in an investigation of the influence of cortisol and related steroids on the differentiation of human mononuclear phagocytes and the phagocytic function of differentiated macrophages are discussed.

of corticosteroid therapy on human monocyte function N Engl J Med *292* 236–241 1975

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8 *Weston W L, Mandel M S Krueger G G & Claman H N* Differential suppressive effect of hydrocortisone on lymphocytes and mononuclear macrophages in delayed hypersensitivity of guinea pigs. J Invest. Dermatol. *59* 345–348 1972

9 *Yu D T Y Clements P J & Paulus H E* Human lymphocyte subpopulations effects of corticosteroids. J Clin. Invest. *53* 565–571 1974

10 *Odegaard A* Functional alterations induced by chlorpromazine in mononuclear blood cells cultured *in vitro* Acta path. microbiol. scand. Sect. C, *83* 59–73 1975

11 *Odegaard A Viken K E. & Lamvik, J* Structural and functional properties of blood monocytes cultured *in vitro* Acta path. microbiol scand Sect. B *82* 223–234 1974

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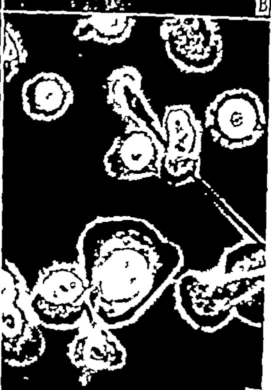
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A

B

C

D

MATERIALS AND METHODS

Cell Culture

The mononuclear cells were separated as described previously (15) The cell suspensions were adjusted to 3×10^6 cells per ml and dispensed on coverslips (11 × 35 mm) in volumes of ½ ml. Two coverslips were placed in each petri dish, diameter 5 cm (Nunclon, Denmark) After 90 minutes of incubation, the medium containing non-adhesive cells was removed, and 2.5 ml of fresh complete medium was added (21) The steroids to be added were treated as described before (15)

Radio-labelling of Candida albicans

Heat killed Candida albicans were labelled with ^{125}I by means of electrolysis (13)

*Test Procedure 2**

The cells were cultured 8 days before testing without addition of drugs. The medium was changed after 1 day and 4 days.

a) Engulfment To each petri-dish 2.5 ml of culture medium containing 2×10^6 ^{125}I labelled *Candida albicans* per ml was added together with the steroid. The culture dishes were incubated at 37 C for 15 minutes, the cells were harvested and rinsed 6 times in Hanks BSS The radioactivity per coverslip was counted in a Wallac GM γ-scintillation-counter (14 22)

b) Digestion. Macrophages, cultured for 8 days *in vitro* on coverslips were used. 2.5 ml complete medium containing 2×10^6 radiolabelled *Candida albicans* per ml was added to each petri dish and incubated at 37 C for 15 minutes. The coverslips were then picked up rinsed 6 times in complete medium, placed in new petri dishes, to which 2.5 ml complete medium was added. After 1 hour the steroids in different concentrations were added. 24 hours later the cells were harvested the medium transferred into plastic tubes and centrifuged at 2000 × *G* for 10 minutes. The supernatants were collected separately The two coverslips, the cell free medium and the sediment were then registered for radioactivity (14 22)

The effect of cortisol on engulfment and digestion of *Candida albicans* was also tested by pre-incubating the cells with cortisol in different concentrations for half an hour before the test started The experiments were then carried out as described above except that cortisol was added during the engulfment of *Candida* and again immediately after transfer of the coverslips to new petri dishes

* Cellular adhesiveness, rosette-forming ability of lymphocytes and survival of macrophages after exposure to different steroids were tested according to *test procedure 1* as described previously (15)

The engulfment was measured as the total activity per petri dish, and the digestion capacity as the percentage of the total radioactivity found in the cell free portion of the medium.

This experiment was also carried out using heat inactivated sera. (56 C for half an hour)

Test Procedure 3

After the first 90 minutes of incubation, complete medium containing different concentrations of steroids were added to the culture dishes. The same procedure was repeated at the medium-shifts after 1 day and 4 days.

a) Cell numbers Cell numbers on the coverslips were counted in an inverted phase contrast microscope at the 4th and the 8th day of culturing

b) Cell function. After 8 days of culturing the medium containing steroid was removed and 2.5 ml fresh culture medium containing 2×10^6 radiolabelled *Candida* particles per ml was added to each petri dish. After incubation for 15 minutes at 37 C the coverslips were picked up, rinsed in culture medium and placed in new petri dishes 2.5 ml culture medium without steroids was added per culture dish. 24 hours later the cells were harvested as described under "Test procedure 2, digestion" The total activity per petri dish will reflect the amount of *engulfed* yeast particles per 2 coverslips. The *digestion capacity* was registered as described earlier

c) Cell morphology Cells treated with different concentrations of steroids were registered morphologically using a microchamber technique described in an earlier paper (21) Photographs were taken using an automatic camera (Ortomat, Leitz, W G) placed on a Leitz phase contrast microscope

Statistics

The experiments were performed in duplicate or triplicate culture dishes. The mean values from each concentration were calculated, and the results from each experiment were expressed as percent

Fig 1 Microphotographs of macrophages cultured for 8 days with different concentrations of cortisol in the medium (Test procedure 3)

A) Cortisol 10^{-1} mg/ml Note small scattered cells with poor granulation and folding of the cell membrane.

B) *Cortisol 10 mg/ml As A but less prominent alterations.*

C) Cortisol 10 mg/ml and

D) Control Normal macrophage appearance (Phase contrast × 1295)

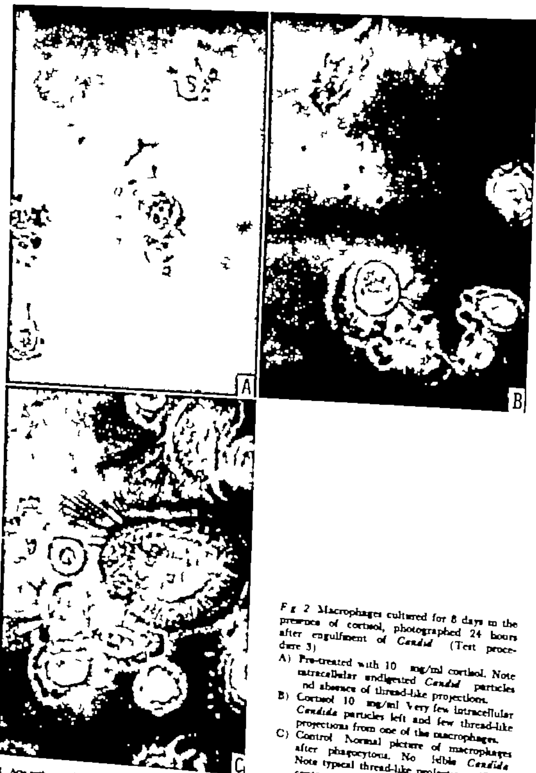


Fig. 2 Macrophages cultured for 8 days in the presence of cortisol, photographed 24 hours after engulfment of *Candida* (Test procedure 3)

A) Pre-treated with 10 mg/ml cortisol. Note intracellular undigested *Candida* particles and absence of thread-like projections.

B) Cortisol 10 mg/ml Very few intracellular *Candida* particles left and few thread-like projections from one of the macrophages.

C) Control Normal picture of macrophages after phagocytosis. No visible *Candida*. Note typical thread-like projections. (Phase contrast × 1295)

TABLE 1 *The Effect of Cortisol on the Differentiation of Monocytes to Macrophages*

Drug conc. mg/ml	Number of cells left after 4 days	Number of cells left after 8 days	Engulfment capacity	Digestion capacity
10^{-1}	79 ± 9	43 ± 10	57 ± 14	54 ± 10
10^{-2}	82 ± 10	51 ± 14	64 ± 17	61 ± 6
10^{-3}	90 ± 9	56 ± 14	62 ± 20	64 ± 4
10^{-4}	89 ± 12	61 ± 14	69 ± 14	75 ± 6
10^{-5}	98 ± 5	79 ± 11	86 ± 15	9[illegible] ± 8
0*	100*	100*	100*	100*

The values listed are the means ± SD of the results from 7 experiments performed in triplicate petri dishes, and the results are expressed as per cent of controls.

* Control without drug addition Number of cells left after 4 days 273 ± 81 range 135–351 × 10^3 cells per culture dish Number of cells left after 8 days 166 ± 49 range 95–228 × 10^3 cells per culture dish. Engulfment capacity 84 ± 38 range 30 133 × 10^3 ct/min, radioactivity per culture dish Digestion capacity 58 ± 3 range 54–61 per cent radioactivity found in the medium.

ages of control values. The figures presented are the mean values from 5-7 experiments ± standard deviation (SD) The p-values were calculated using the Wilcoxon Two-Sample test.

RESULTS

The effect of different steroids on macrophages cultured 8 days *in vitro* in the absence of drug was tested. Within the concentrations 10^{-5} to 10^{-1} mg/ml cortisol, testosterone desoxycorticosterone and tetrahydrocortisol had no significant effect, neither on engulfment nor on digestion of yeast particles, tested as described as test procedure 2

Pre-incubation for half on hour with cortisol reduced the engulfment of *Candida* slightly but this effect was not significant ($p > 0.05$) Cortisol being present in the medium half an hour before the experiment and onwards during the engulfment phase as well as the digestion phase of phagocytosis did not influence the digestion ability of the macrophages. Heat inactivation of the sera used in the experiments did not change these findings.

In the experiments carried out as described as test procedure 3 the steroids were added at the times of medium shifts 90 minutes, 1 day and 4 days after culture start. Cortisol had a strong and dose dependent suppressing effect on the differentiation of monocytes to macrophages, measured as the ability of the remaining cells to digest *Candida albicans* ($p < 0.01$ in concentrations of 10^{-4} to 10^{-1} mg/ml) and judged on the basis of the morphology of the cells left on the coverslips as seen in phase contrast microscopy The number of cells left after 8 days in culture showed a dose dependent decrease to about 40 per cent of the control values at the concentration of 10^{-1} mg/ml of cortisol (Table 1) ($p < 0.01$)

The engulfment of radiolabelled *Candida albicans* measured as radioactivity per coverslip was also reduced to about 60 per cent (Table 1) These values must be considered together with the number of cells left on the coverslips. Correction for the decline in cell number is not done The decreased engulfment capacity thus expresses merely the reduction in the number of cells per coverslip.

In the digestion experiments however the results are quite independent of the number of cells per coverslip and the dose dependent reduction of the ability to digest the engulfed *Candida albicans* to about 50 per cent of the control values thus reflects the functional state of the cells left on the coverslips (Table 1)

The microphotographs of cells after cortisol treatment according to test procedure 3 revealed that the cells were less differentiated

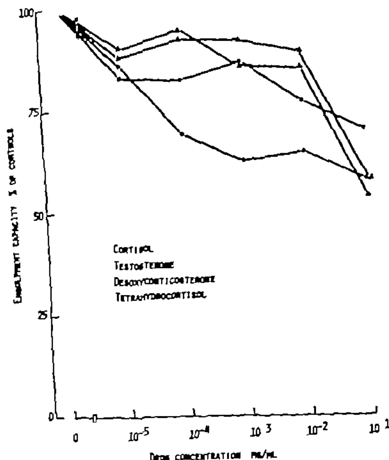


Fig. 4 Effect of different steroids on the engulfment capacity of macrophages after 8 days exposure to the drug. The results are expressed as in Fig. 3

TABLE 2. *The Effect of Testosterone on the Differentiation of Monocyte to Macrophages*

Drug conc. mg/ml	Number of cells left after 4 days	Number of cells left fter 8 days	Engulfment capacity	Digestion capacity
10	44 ± 22	36 ± 24	69 ± 18	47 ± 24
10^{-2}	95 ± 4	79 ± 16	76 ± 12	82 ± 19
10	97 ± 1	94 ± 10	86 ± 20	93 ± 14
10^{-}	98 ± 3	104 ± 17	82 ± 12	95 ± 17
10	100 ± 2	102 ± 11	83 ± 15	96 ± 18
0*	100*	100*	100*	100*

5 experiments. For explanations of the figures see Table 1

Control without drug addition. Number of cells left after 4 days 248 ± 63 range 159–342 × 10³ cells per culture dish. Number of cells left after 8 days 78 ± 13 range 63–98 × 10³ cells per culture dish. Engulfment capacity 100 ± 35, range 66–151 ct/min, radioactivity per culture dish. Digestion capacity 62 ± 12, range 50–78 per cent radioactivity found in the medium.

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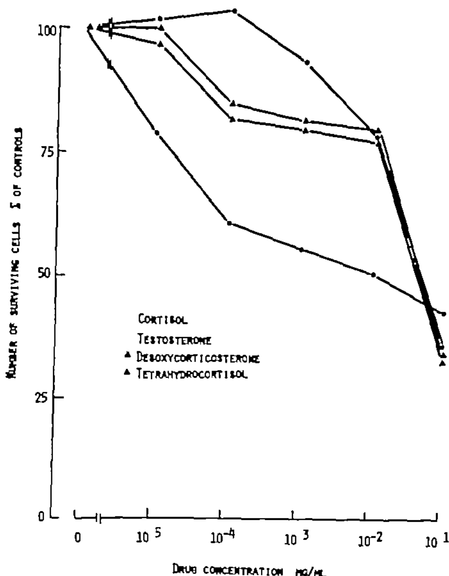


Fig 3 Effect of different steroids on the number of cells left after 8 days exposure to the drug. The values plotted are the means of the results from 7 experiments using cortisol and 5 experiments using the other steroids expressed as per cent of controls.

in the cultures exposed to cortisol in doses exceeding 10^{-4} mg/ml demonstrated as reduced size of the cells and reduction of perinuclear granulation in other words a reduced development of lysosomes (Fig 1) One day after engulfment of *Candida albicans* the yeast particles could still be seen within the cytoplasm of the cells treated with high doses of cortisol (Fig 2)

The effect of testosterone, tested according to test procedure 3 was somewhat different from that of cortisol The number of cells left on the coverslips decreased suddenly between the concentration of 10^{-4}-10^{-1} mg/ml, indicating a toxic effect upon the cells (Fig 3) The effect on the engulfment and the digestion capacities followed the same trend (Table 2)

Desoxycorticosterone and tetrahydrocortisol, tested in the same way caused in principle the same alterations as testosterone The cells left on the coverslips after treatment with 10^{-1} mg/ml of these steroids showed, however an improved ability to digest the engulfed yeast particles, as compared with cortisol and testosterone (Fig 3 4 5)

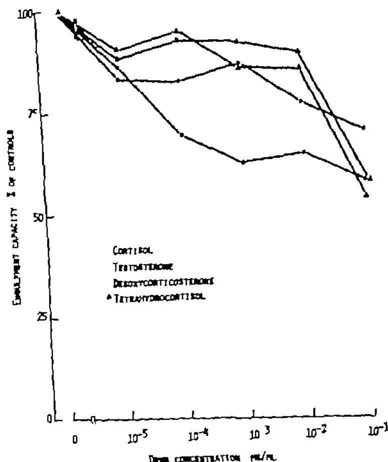


Fig 4 Effect of different steroids on the engulfment capacity of macrophages after 8 day exposure to the drug The results are expressed as in Fig. 3

TABLE 2 *The Effect of Testosterone on the Differentiation of Monocytes to Macrophages*

Drug conc. mg/ml	Number of cells left after 4 days	Number of cells left after 8 days	Engulfment capacity	Digestion capacity
10⁻	44 ± 22	36 ± 24	69 ± 18	47 ± 24
10⁻²	93 ± 4	79 ± 16	76 ± 12	82 ± 19
10	97 ± 1	94 ± 10	86 ± 20	93 ± 14
10⁻	98 ± 3	104 ± 17	82 ± 12	95 ± 17
10	100 ± 2	102 ± 11	83 ± 15	96 ± 18
0*	100*	100*	100*	100*

5 experiments For explanations of the figures see Table 1

Control without drug addition Number of cells left after 4 days 248 ± 65, range 159 342 × 10^{3} cells per culture dish. Number of cells left after 8 days 78 ± 13 range 63–98 × 10 cells per culture dish Engulfment capacity 100 ± 35, range 66–151 ct/min, radioactivity per culture dish. Digestion capacity 62 ± 12, range 50–79 per cent radioactivity found in the medium

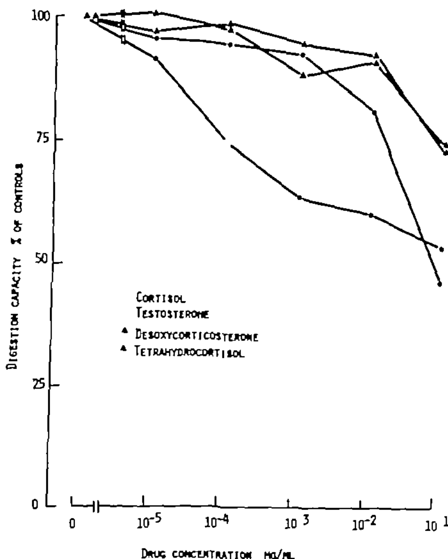


Fig 4 Effect of different steroids on the digestion capacity of macrophages after 8 days exposure to the drug The results are expressed as in Fig 3

Morphologically the cells treated with testosterone desoxycorticosterone and tetra hydrocortisol showed only small changes in the differentiation within a concentration range from 10^{-5} 10^{-2} mg/ml In the highest concentration however the cells were small often rounded and vacuolated 24 hours after addition of *Candida* yeast particles could be observed within the cytoplasm in those cells treated with the highest concentration of these steroids.

DISCUSSION

In concentrations up to 10^{-1} mg/ml, none of the steroids tested had any significant effect on the different steps of phagocytosis. Pre-treatment of the cells with steroids for 8 days before testing however resulted in a dose dependent reduction of the phagocytic function of cells treated with cortisol. The other steroids tested did not show this effect. The morphology of the cells treated with various doses of cortisol indicated a dose-dependent inhibition of the differentiation of monocytes to macrophages, and a reduced survival of the exposed cells.

Many reports deal with the effect of glucocorticosteroids on phagocytosis. Experiments carried out on separated lysosomal fractions from liver cells have shown that cortisol has a stabilizing effect on the lysosomal membrane measured as reduced release of proteo-

lytic enzymes (16, 17). This effect has also been demonstrated in leucocyte suspensions containing mostly granulocytes (20). A stabilization of the lysosomal membrane could impair the fusion between lysosomes and phagosomes. There is, however, a discrepancy between the results from these experiments and the findings by various authors who have tested the phagocytic ability of different kind of macrophages *in vitro*. Intracellular killing of *Staphylococcus albus* and other bacteria (except *Salmonella typhimurium*) in mouse peritoneal macrophages was not altered by cortisol (12). Neither did cortisol inhibit phagocytosis of antibody-coated erythrocytes in doses below 10^{-1} mg/ml (6) nor the uptake and digestion of heat-agglutinated serum albumin and *Salmonella typhimurium* in doses below 5×10^{-4} M (1 mg/ml) (19). In the two latter experiments, an inhibition of the uptake was found in doses exceeding 10 mg/ml of cortisol. The digestion capacity however was not altered (12, 19). Studies of human monocytes revealed no effect on the uptake of cryptococci, though the bactericidal activity was reduced during the treatment (9). These findings are in accordance with those obtained by us, using phagocytosis of *Candida albicans* by human macrophages as test model, and indicate no direct effect of cortisol in doses below 10^{-} mg/ml on the uptake of particles or on the fusion of phagosome and lysosome, necessary for the digestion of the ingested particles (12).

Binding of cortisol to cytosol receptors may be the explanation of the lacking effect of cortisol on lysosomes in intact cells. After diffusion through the cell membrane these receptors pick up cortisol and transport it to a nuclear receptor (7). In the nucleus, cortisol has been found to interfere with the gene repressor complex and thus the transcription of DNA into RNA (8, 11).

The strong dose dependent, depressing effect of cortisol on the differentiation of monocytes to macrophages observed after culturing the cells in the presence of cortisol from 90 minutes to 8 days was the most prominent finding in our study. This differentiation consists of a tenfold increase of the cytoplasm of the cells and an increased production of lysosomes (4, 21). Of course this process is dependent on a considerable protein synthesis which is known to be inhibited directly or indirectly by cortisol (8). The catabolic effect of this steroid can therefore explain the reduced survival, the diminished size of the cells and the reduced phagocytic function found after long-term treatment with cortisol in the cell cultures.

This effect was not found by testing the other related steroids. In these cases, doses up to 10^{-4} mg/ml had no or little effect on the cells. In a concentration of 10^{-} mg/ml a toxic effect parallel with decreased survival, was observed and a few small, vacuolated cells with poor phagocytic function were left on the coverslips.

Cortisol has been found to reduce the number of macrophages at the site of inflammation (18). The number and efficiency of macrophages are crucial for the outcome of an infection. Clinical investigation and animal experiments have shown that treatment with immunosuppressive drugs such as cortisol reduce the host resistance against various infective agents. So far the explanation of the reduced number of macrophages at the site of inflammation during cortisol treatment is not known. van Furth (12) has shown that, during cortisol treatment of mice, a moderate, impaired release of monocytes into the circulation and a slight decrease in production of monocytes cannot explain the fall in circulating monocytes which occurs during cortisol treatment. Since there is no clear evidence of an alteration of the vascular wall during treatment with cortisol he assumes that a sequestration of monocytes is the main cause of the fall in circulating monocytes and the reduced number of monocytes at the site of inflammation.

The cortisol induced inhibition of the differentiation of monocytes to macrophages and the reduced survival found in this study may serve as an additional explanation of the effect of cortisol on the mononuclear phagocytes.

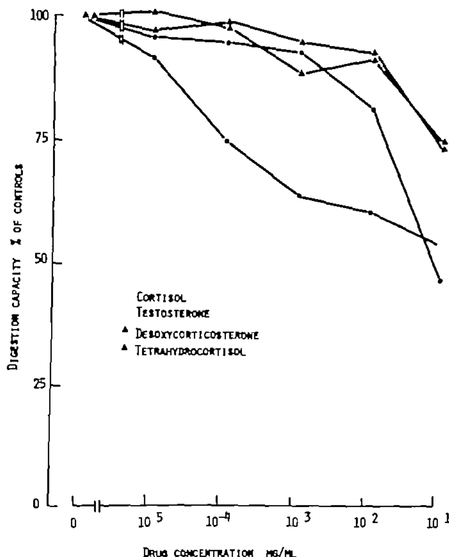


Fig 5 Effect of different steroids on the digestion capacity of macrophages after 8 days exposure to the drug The results are expressed as in Fig 3

Morphologically the cells treated with testosterone desoxycorticosterone and tetra hydrocortisol showed only small changes in the differentiation within a concentration range from 10^{-4}–10^{-2} mg/ml. In the highest concentration however the cells were small often rounded and vacuolated. 24 hours after addition of *Candida* yeast particles could be observed within the cytoplasm in those cells treated with the highest concentration of these steroids.

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In concentrations up to 10^{-1} mg/ml, none of the steroids tested had any significant effect on the different steps of phagocytosis. Pre treatment of the cells with steroids for 8 days before testing however resulted in a dose dependent reduction of the phagocytic function of cells treated with cortisol The other steroids tested did not show this effect. The morphology of the cells treated with various doses of cortisol indicated a dose-dependent inhibition of the differentiation of monocytes to macrophages, and a reduced survival of the exposed cells.

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THE INTERPLAY BETWEEN TARGET ORGAN CONCENTRATIONS OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS AND CELL MEDIATED IMMUNITY IN BABY MICE

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Marker O Andersen, G Thorner & Volkert, M. The interplay between target organ concentrations of lymphocytic choriomeningitis virus and cell mediated immunity in baby mice Acta path. microbiol. scand. Sect. C, 84 23–30 1976

Circumstantial evidence has been presented which supports the view that the fatal LCM virus infection is due to an immunological conflict in the host animal Hitherto, this outcome of the infection has only been observed in intracerebrally infected mice. In the present study the intraperitoneal infection in young mice was investigated and the results revealed a new example of this immunological conflict. In mice infected a few days after birth, concentrations of the virus in the brain are high, while the CMI response is non-measurable. If the infection is induced when the mice are 28 days old or more, there is little virus in the CNS but a strong CMI response can be demonstrated. All the mice in these two age groups survive. If mice are infected when they are 17–19 days old, however they raise a moderate CMI response nine days after infection and, at the same time, their brains contain virus in high titres. The mortality among mice infected at this age is 100 per cent, indicating that this combination is fatal. The lives of these animals can be saved by anti theta serum or if they are transplanted with syngeneic lymphoid cells sensitized to LCM virus. Our results strongly suggest an interplay between, on the one hand, the spread and the magnitude of the virus infection in the brain and, on the other the cell mediated immune response. This interplay seems to be decisive for the clinical outcome of the LCM infection in mice

Key words Lymphocytic choriomeningitis virus cell mediated immunity target organ concentrations.

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When LCM virus is introduced into the newborn mouse a persistent virus carrier state is established, no matter the route of administration. In the mature animal, however the infection leads to death if the virus is given intracerebrally and to permanent immunity if it is injected into the peritoneal cavity These features of the LCM virus infection in the mouse have been demonstrated by Traub (19) and by other workers (6, 9)

It is generally agreed that the disease in-

Technical assistance from Mrs. *B H Hansen* is gratefully acknowledged I am also very grateful to Prof *J Lamvik* and Prof *K B Eik-Nes* for critical discussions and help during the course of this study and the preparation of the manuscript.

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REFERENCES

1 *Anderson R J Schafer L. A & Olin D B* Infectious risk factors in the immunosuppressed host. Am. J Med *54* 453–460 1973

2 *Bach M C Sahyoun A Adler J L Schlesinger R M Breman J Madras P Peng Fang-ku & Monaco A P* Influence of rejection therapy on fungal and nocardial infections in renal transplanted recipients. Lancet *I* 180–184 1973

3 *Bovornkitti S Kangsadal P Sathirapat S & Oonsombatti P* Reversion and reconversion rate of tuberculine skin reactions in correlation with use of prednisone Dis. Chest. *38* 51–55 1960

4 *Cohn Z A & Wiener E* The particulate hydrolases of macrophages. I Comparative enzymology isolation and properties. J Exp Med *118* 991–1008 1963

5 *Fauce R M & Pierce-Chase C H* Comparative effects of corticosteroids on host resistance to infection in relation to chemical structure J Exp Med. *125* 807–821 1967

6 *Gemsa D Fudenberg H H & Schmid R* Erythrophagocytosis Regulation of the heme-degrading enzyme system. In Wagner W H and Hahn, H (Ed) Activation of macrophages, 1 ed. vol 2 Excerpta Medica, Amsterdam 1974 p 178–189

7 *Munck A & Brinck Johnsen T* Specific and non-specific physico-chemical interactions of glucocorticoids and related steroids with rat thymus cells in vitro J Biol Chem. *243* 5556–5565 1968

8 *Myles A B & Daly J R* Corticosteroid and ACTH treatment 1 ed Edward Arnold (publishers) Ltd London 1974 p 46–52

9 *Rinehart J J Sagone A L Balcerzak S P Ackerman G A & Lo Buglio A F* Effects of corticosteroid therapy on human monocyte function. N Engl J Med 292 236–241 1975

10 *Thomson J & van Furth R* The effect of glucocorticosteroids on the kinetics of mononuclear phagocytes. J Exp. Med *131* 429-442 1970

11 *Tomkins G M & Gelehrter T D.* The present status of genetic regulation by hormones. In Litwach, G (Ed.): Biochemical Actions of Hormones, vol II Academic Press, London and New York 197? p 1–17

12 *van Furth R* The effect of immunosuppressive drugs on the formation and kinetics of mononuclear phagocytes. In Wagner W H and Hahn, H. (Ed) Activation of macrophages, 1 ed. vol. ? Excerpta Medica, Amsterdam 1974 p 178–189

13 *Løken A E* ¹²⁵I Labelling of *Candida albicans* by electrolysis. Acta path. microbiol. scand. Sect. B, *82* ?19 222 1974

14 *Løken A E & Odegaard A* Phagocytosis of heat killed radiolabelled *Candida albicans* by human blood monocytes cultured *in vitro*. Acta path. microbiol. scand Sect. B, *82* 235–244 1974

15 *Løken A E* The effects of steroids on adhesiveness, rosette forming ability and survival of cultured human mononuclear cells. Acta path microbiol scand Sect. C, *84* 5–12, 1976

16 *Weissmann G & Thomas L* Studies on Lysosomes I The effect of endotoxin, endotoxin tolerance and cortisone on the release of acid hydrolasis from a granular fraction of rabbit liver J Exptl Med. *116* 433–450, 1962.

17 *Weissmann G & Thomas L* The effect of corticosteroids on connective tissue and lysosomes. Recent Progr Hormone Res. *20* 215–245 1964

18. *Weston W L Mandel M S Krueger G G & Claman H N* Differential suppressive effect of hydrocortisone on lymphocytes and mononuclear macrophages in delayed hypersensitivity of guinea pigs. J Invest. Dermatol. *59* 345–348 1972

19 *Wiener E Marmary Y & Curelaru Z Z.* The in vitro effect of hydrocortisone on the uptake and intracellular digestion of particulate matter by macrophages in culture. Lab. Invest. 20 220–226 1972

20 *Wright D G & Malawista S E* Mobilization and extracellular release of granular enzymes from human leucocytes during phagocytosis Inhibition by colchicine and cortisol, but not by salicylate Artritis Rheum. *16* 749–758 1973

21 *Odegaard A Løken A F & Lamvik J* Structural and functional properties of blood monocytes cultured *in vitro* Acta path. microbiol scand. Sect B *82* 223 234 1974

22 *Odegaard A* Functional alterations induced by chlorpromazine in mononuclear blood cells cultured *in vitro* Acta path. microbiol. scand Sect. C, *83* 59 73 1975

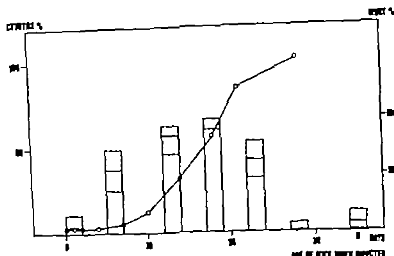


Fig. 1. Development of cell mediated immunity to LCM virus in C3H mice of varying age (bars). The dotted lines on the bars represent 99 per cent confidence limits. N: mortality of uninfected C3H babies. The cytotoxicity assays were carried out nine days post infection.

mune mice serving as donors were mice either in the early or the late state of the LCM viral infection. The animals were infected by i.p. inoculations of 10^3 LD_{50} LCM virus. Early immune cells were harvested from the spleen of mice nine days post infection, late immune cells were harvested from the same organ 30 days post infection. The preparation of the cell suspensions has been described previously (20); the cells were transplanted by i.p. injections.

Cell mediated immunity (CMI). The CMI was determined as the in vitro cytotoxicity of single cell suspensions of spleen cells from LCM virus infected C3H mice upon syngeneic LCM virus infected L cells. The cytotoxicity in this test was evaluated by measuring the amount of Cr released from the labelled L cells during an 18 h incubation with the sensitized lymphocytes. It was expressed as cytotoxic index. The test and the calculation of the index has previously been described in detail (13, 14).

RESULTS

Life Expectancy

Preliminary results showed that a certain age can be considered critical to C3H mice with regard to life expectancy following i.p. inoculation of 1×10^3 LD_{50} LCM virus. In order to analyse this phenomenon, six groups of mice were infected as mentioned. The number of mice in each group varied between 40 and 60. The age at which infection was induced and the mortality percentage in the various groups are illustrated by the columns presented in Fig. 1. It is seen from this figure that the rate of mortality among C3H baby mice infected with 1×10^3 LD_{50} LCM virus at birth is low and not different from that of uninfected animals. With increasing age at the time of infection the mortality also increases until the age of 17–19 days, when it amounts to 99 per cent. If the animals are a few days older at the time of infection, the mortality decreases again and all mice infected at the age of 28 days, will survive.

A calculation of the average time of death in the different groups showed that there was no difference between any of the groups as to the interval of time between infection and death. The distribution of the deaths over different days following infection of all mice which died in this experiment is shown in Fig. 2. It is seen that the variation is great, but that there is a definite maximum on day 12.

Cell Mediated Immunity

In order to investigate the development with increasing age of CMI expressed as specific cytotoxicity of lymphocytes from the infected animals against syngeneic LCM virus infected target cells, the following experiments were carried out. Groups of ten mice, each group representing a different age, were given 1×10^3 LD_{50} LCM virus i.p. nine days before the cytotoxicity was assayed. In this

volving the central nervous system (CNS) and seen to follow LCM virus infection is due to the effect of the immune response of the host animal This knowledge is mainly based on experiments in which it has been shown that various immunosuppressive treatments can save the lives of otherwise lethally infected animals (4 10 1 18 7 17 8) Further observations from different laboratories strongly indicate that a short lived T lymphocyte population rich in cytotoxic cells is the key factor in the fatal disease caused by LCM virus (3 5 21) It has recently been shown in our laboratory that death to occur after intracerebral (i.c.) infection of the adult mouse can be prevented by transfer of such cells from syngeneic animals (21) The cells concerned may thus be beneficial as well as detrimental to the host and a factor other than this must therefore concurrently contribute to the clinical outcome of the infection

Based upon the study of the various above mentioned types of infection it could be assumed that the cytotoxic T cells were dangerous to the host only if the cell mediated immunity (CMI) had been raised at a time when the brain was virus infected since this combination has hitherto been seen only in the intracerebrally infected adult mouse In the search for a feasible model in which this assumption could be investigated, preliminary experiments showed that young mice during immunological maturation after birth gradually develop the ability to raise a CMI response to LCM virus from unresponsiveness to the level of adult mice

Since it has been demonstrated that the CMI response has a reducing capacity on LCM virus titres in many organs of the infected animal (15 21) it could be expected that the study of young mice during immunological maturation would reveal many different levels of CMI response parallel with varying degrees of virus infection in the brain

The present paper describes experiments in which the interplay between the magnitude of the CMI the LCM virus infection in the brain and the life expectancy of the host animal were studied in intraperitoneally LCM infected mouse babies of the C3H strain.

MATERIAL AND METHODS

Mice Except for virus titration purposes, highly inbred C3H mice were used throughout the study

Lymphocytic choriomeningitis (LCM) virus. The LCM virus originated from Dr *E Traub* (Ludwig-Maximillians Universität München, Germany) For infection of mice or L cells, which served as target cells in the cytotoxic reaction virus propagated in tissue culture (L cells) was employed. This virus stock was produced and stored as described previously (14)

Virus titrations were carried out by i.c. inoculations into ordinary 12–14 g white Swiss mice When the virus content of brains was determined, these organs were titrated individually Whole brains were grinded thoroughly with sterile sea sand and phosphate buffered saline (PBS) When the suspension had been centrifuged, the supernatant was diluted in PBS and titrated in 10-fold dilutions. Titration end points were calculated by the *Kärber* method (11) and titres were expressed as log 10 LD_{50}/0.03 ml intracerebral dose

Survival experiments When the mortality of virus infected mice was determined in baby mice and in adolescent mice of the C3H strain, the animals were given 10^3 LD_{50} LCM virus intraperitoneally (i.p.) at different times after birth. All siblings in one litter received the same treatment and deaths were recorded daily The observation period covered from four to five weeks, except in experiments in which the protective effect of anti-theta serum was investigated In these experiments the observation time covered only three weeks, because only one single injection of the preparation was given

X irradiation The irradiation was administered by a Siemens Stabilipan therapy machine operated with the following factors 200 kV 15 mA, 1.0 mm copper filtration The dose rate was 46 R per min, and half value layer was 1.5 mm of copper

Anti-theta serum The preparation was kindly supplied by Dr *Bent Rubin* (Statens Seruminstitut Copenhagen) It was produced by immunizing mice of the AKR strain with C3H mouse thymocytes The detailed characteristics of this serum have been given in a previous paper (21)

Chemical immunosuppressives Cyclophosphamide Endoxan Asta® 100 mg (ASTA Werke W Germany) was dissolved in PBS less than one hour before injection Hydrocortisone Hydrocortisat® Leo (LEO Pharmaceutical Company Copenhagen) contains 25 mg hydrocortisone acetate per ml. Methotrexate Methotrexate Sodium (Lederle Laboratories Division, USA)

Transplantation of immune syngeneic cells Im-

Fig. 3 The development of brain virus titres in C3H mice i.p. inoculated with LCM virus at an age of less than 24 hours (O—O) 19 days (▲—▲) and 40 days (□—□) respectively

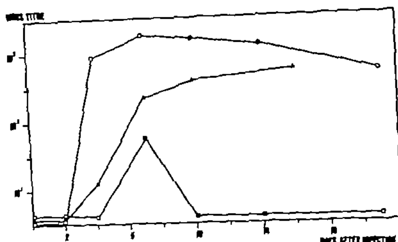


TABLE 1 *The Effect of Various Immunosuppressive Treatments on the Mortality of C3H Mice i.p. Infected with LCM Virus at an Age of 17–19 Days*

Treatment	Dose	Day*	Virus	Dead/ I jected	Mortality %
Anti-theta serum	0.2 ml i.p.	−2	1000 LD_{50}	0/10	0
	0	–		10/13	77
X-irradiation	100	−1		25/25	100
	100 r	–	0	0/16	0
Cyclophosphamide	150 mg/kg i.p.	3	1000 LD_{50}	51/51	100
		3	0	0/10	0
Methotrexate sodium	1 mg/kg c.	0–2–4	1000 LD_{50}	23/24	96
			0	3/19	16
Hydrocortisone acetate	40 mg/kg c.	1 3–6	1000 LD_{50}	23/24	96
Early immune cells	100×10^6 i.p.	0	1000 LD_{50}	0/9	0
		2		10/10	100
Late immune cells		0	1000 LD_{50}	7/8	88

The virus infection was always carried out on day 0.

takes place more slowly and the titre level is below that of the virus carriers.

The Effect of Various Treatments on the Mortality of i.p. Infected Adolescent Mice

Various immunosuppressive treatments were applied to C3H baby mice infected i.p. with 1×10 LD_{50} LCM virus at an age of 17 to 19 days. The treatments, the dosages, and the effect on the survival of the mice are listed in Table 1. As seen in this table none of the physical or chemical immunosuppressive treatments had any effect on the survival rate. On the contrary treatment with anti-theta serum had an obvious life-saving effect. Table 1 records also the effect of transfer to the infected baby mice of spleen- and lymph node cells from syngeneic, adult animals which had been inoculated with LCM virus i.p. nine and 40 days earlier. The data presented demonstrate clearly that the early immune lymphoid cells prevented death completely when given i.p. in a number of 100×10^6 on the day of the virus infection which, without treatment, would have led to the death of the animals. If the cells were given later in the infection, however the

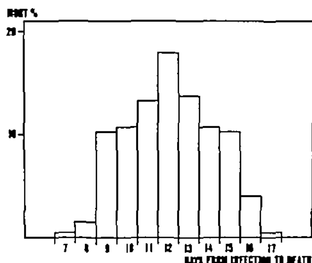


Fig 2 Mortality among young C3H mice on different days following i.p infection with LCM virus.

way the level of CMI in mice in all the groups was measured in one experiment, using one batch of infected target cells and under the same conditions. This time interval between infection and assay was chosen because previous experiments had shown that the maximal cytotoxic effect was demonstrable in lymphocyte preparations from mice which were inoculated with virus nine days before the test In each of the experiments in this series lymphocytes from normal adult mice which had also received 1×10^3 LD_{50} of LCM virus nine days earlier served as controls. With a view to comparing the results obtained in various experiments of this kind the cytotoxicity of the cells from the individual age groups were *in each separate* experiment expressed as percentages of these positive controls At the time of the cytotoxic assay the CF antibody level in the sera of the mice was also determined

The averaged results from three of these experiments are depicted in Fig 1 It is seen from this figure that, if the mice were infected i p with the virus before the age of ten days, the CMI level nine days later would be non measurable by our method. From this point, the cytotoxicity rises gradually until the age of 28 days, when the degree of cytotoxicity is indistinguishable from that in older mice. None of the mice had CF antibodies nine days after an i p infection. As mentioned above, the data obtained in the mortality experiment are also included in Fig. 1 The collocation of these results shows that, while the CMI is at a very low level the mortality is low too Likewise when the CMI has reached levels otherwise seen in adult mice all the animals survive the infection. When the CMI is supraminimal or inframaximal, however the mortality is high, reaching a maximum of 100 per cent when the mice are infected at the age of 17–19 days, at which time a cytotoxic activity of approximately 50 per cent of the level in adult mice is demonstrable.

Virus Concentrations in the Brain

It was investigated whether there was any relationship between mortality and not only the CMI response but also the degree of virus infection of the brain The experiments with this aim in view were carried out as follows three groups of mice were infected i p at the ages of 0 19 and 40 days, respectively Each group consisted of 25 mice. At different times after the infection, five mice from each group were sacrificed, and the virus content of the brains was titrated.

The results obtained in this experiment are shown in Fig 3 which clearly demonstrates that mice infected during the first 24 hours after birth within four days develop a high virus titre in the brain, and that this titre decreases only slightly during the first three weeks following infection. In fact, the virus content of the brains of these animals remains at a high level for the rest of their lives, i.e. they become virus carriers. When adult (40 days old) C3H mice are infected in the same way the maximal titres are low in comparison and are only reached after seven days. Ten days after infection the virus has disappeared again from the brain of these animals. The time course of the LCM virus infection in animals inoculated at an age of 19 days is rather similar to the one seen following infection of newborns. The titres obtained are high, but the increase of the titre

suppress the growth of the virus when it has reached the CNS. The CMI response is not insignificant, however, since it amounts to about 50 per cent of the response in the adult mice. Thus, once again a situation is created in which the simultaneous presence of early and cytotoxic immune cells, on the one hand, and high virus concentrations in the brain, on the other, can create a fatal immunological conflict as it is seen in the i.c. infected adult mouse.

On closer inspection of the graph depicting the development of the CMI response (Fig. 1), it might seem surprising that the mortality is not insignificant at the age of eight days, even though, at this age, the lymphocytes from these animals show no detectable cytotoxicity nine days after infection. It might also seem somewhat puzzling that the mortality at 80 per cent CMI response is 63 per cent, while all the mice survive when their lymphoid cells reach an "adult" level (100 per cent) of cytotoxicity. This pronounced S-shape of the curve, however, can be explained on the basis of technical factors. As regards the low end, it could be assumed to be the result of a lack of sensitivity in our assay system and, as regards the upper end, the technical necessity of using only one ratio between lymphocytes and target cells may be responsible for difficulties to arise when different levels of very high cytotoxicity are to be distinguished.

As mentioned above, immunosuppressive treatment has prevented the death of mice with otherwise fatal LCM virus infections. To characterize the here described assumed immunological conflict in this respect, the influence of various immunosuppressants on the mortality of adolescent mice infected when they were 17–19 days old was determined. As accounted for under "Results", anti-theta serum was able to protect completely the infected baby mice. It was also shown that early immune cells, which are known to limit the virus proliferation in different organs in the infected mouse (15), had the same life-saving effect.

The course of the disease leading to the death of the C3H baby mice is significantly protracted as compared with that of i.c. inoculated adult animals. Convulsions have often been observed among the infected baby mice, however, and post mortem, the pronounced extension of the hind legs which is rather characteristic of the LCM death, has frequently been observed. Therefore, we have found no reason to believe that deaths among these baby mice could be due to any other external factor than the LCM virus infection.

Since it is strongly believed that anti-theta serum may attack the cellular immune faculty specifically (21), and since it is beyond doubt that the action of early immune T cells is a direct cellular event, the results presented in this paper are, in our opinion, in support of the assumption that the development of the CMI is one of two decisive factors in the successful immune response to the LCM virus infection in the mouse as well as in the fatal disease due to this infection, and that the virus infection of the CNS is the other.

REFERENCES

1. *Barlow, J. & Hotchin, J.*: Induction of persistent tolerant infection with lymphocytic choriomeningitis virus in adult mice by amethopterin treatment. Ann. St. Rep. N.Y. Dep. Hlth., 1962, p. 39.
2. *Cole, G. A., Gilden, D. H., Monjan, A. A. & Nathanson, N.*: Lymphocytic choriomeningitis virus. Pathogenesis of acute central nervous system disease. Fed. Proc. *30*: 1831–1841, 1971.
3. *Cole, G. A., Nathanson, N. & Prendergast, R. A.*: Requirement for θ-bearing cells in lymphocytic choriomeningitis virus-induced central nervous system disease. Nature, Lond. *238*: 335–337, 1972.
4. *Collins, D. N., Weigand, H. & Hotchin, J.*: The effect of pretreatment with X-rays on the pathogenesis of lymphocytic choriomeningitis in mice. J. Immunol. *87*: 682–687, 1961.
5. *Doherty, P. C. & Zinkernagel, R. M.*: T-cell mediated immunopathology in viral infections. Transplant. Rev. *19*: 89–120, 1974.
6. *Haas, V. H.*: Some relationships between LCM virus and mice. J. Infect. Dis. *94*: 187–198, 1954.
7. *Haas, V. H. & Stewart, S. E.*: Sparing effect of a-methopterin and guanazolo in mice infected with the virus of lymphocytic choriomeningitis. Virology *2*: 511–516, 1956.

transplantation of immune cells had no effect on the death rate among baby mice Immune cells harvested late in the infection and transplanted as the early immune cells had no measurable protective effect

DISCUSSION

Although tissue damage during LCM virus infection in mice has been reported (16) it is well established that the fatal LCM disease is not due to the cytopathic effect of the infecting agent, rather to the host animals own immune response to the virus.

Histological examinations of the brain of i.c. infected adult animals show mononuclear cell infiltrations in the choroid plexus and in the meninges (2) Moreover experiments using fluorescence microscopy have revealed that the infected cells are found in the chorio-meningeal plexus as islands in the parenchyma and to a smaller extent in the nervous tissue (2 12) Furthermore data have ben presented which indicate that the mononuclear infiltrates contain cells with strong cytotoxic activity *in vitro* against syngeneic LCM virus infected target cells (22) Hence it is tempting to assume that one of the pathogenic mechanisms underlying the events leading to the death of the animal parallels the cell mediated cytotoxicity as it is taking place in the *in vitro* assay system.

In the i c infection of the adult mouse the CMI response is already detectable a few days after infection. Subsequently it increases rapidly and death occurs during the rise As to the virus concentrations in brains, they increase very quickly owing to the localization of the virus inoculation The high virus titre and the strong CMI response occur simultaneously and thus, conditions for a fatal immunological conflict are present.

If the adult mouse is infected i p the CMI is also strong and has a characteristic time course the maximum being achieved on day nine (14 13) Contrary to the i c infected mouse however the maximal virus titres in the brain are rather low they are reached only on day seven and on day ten virus has already disappeared again Such animal survives and becomes immune It can therefore readily be assumed that the spread of the virus to the brain in this situation is delayed because of the intraperitoneal route of infection and at the time when virus proliferation in the target organ is about to start, the number of sensitized cytotoxic T lymphocytes is sufficient to suppress the virus infection in the CNS and to prevent it from reaching an extent sufficient for the cell mediated immune reaction to cause severe tissue damage

When newborn mice are infected with LCM virus, the virus titres in the brain reach high levels very fast—no matter the route of administration Such animals are not able to develop cytotoxic T cells specifically sensitized to the infecting agent, however and the mice survive as permanent virus carriers.

The results presented in this paper show a new situation We have found that infection with the Traub strain of LCM virus applied by the intraperitoneal route, which in our laboratory hitherto has been considered harmless to mice nevertheless is dangerous to baby mice and adolescent mice of the C3H strain within a certain range of age When the animals are between 17 and 19 days old, the mortality rate is even 100 per cent.

Furthermore, the data seem to indicate that there is a definite correlation between the magnitude of the CMI response the virus titre in the brain and the life expectancy following i p LCM virus infection of these animals.

On the basis of the above, it is tempting to interpret the results in the present paper as a dead heat between the proliferation of virus in the brain on the one hand and the production of specific early immune T cells, on the other

The time course of the virus proliferation in the adolescent mice i p infected with LCM virus is quite different from that in the adult animals infected in the same way The virus titres in the brains of young mice rise earlier reach higher levels and remain high presumably because the CMI response in these animals is not sufficiently strong to limit and

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FRAGMENTS OF IMMUNOGLOBULINS IN HUMAN FAECES

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Haneberg, B. & Endresen, C. Fragments of immunoglobulins in human faeces. Acta path. microbiol. scand. Sect. C, 84: 31–36, 1976.

Fab-fragments of IgG were easily demonstrated in extracts of faeces from healthy infants and children. Employing immunoelectrophoresis with antisera to whole human serum and to Fab-fragments of IgG, a marked precipitation line, likely to represent such fragments, was evident in the cathodal or γ-region. Usually no precipitate was formed with antisera specific for γ-heavy chains or Fc-fragments of IgG. Presumably IgG in the gut is partially destroyed before being excreted with faeces. Results of immunoelectrophoresis, gel filtration as well as polyacrylamide gel electrophoresis, using antisera to α-heavy chains, indicated the presence of fragments of IgA in some faecal extracts. Fragments of IgA could not be demonstrated in IgA-deficient patients, and no fragments of either class, or only traces of Fab-fragments of IgG were found in agammaglobulinaemic patients. No antibody activity against rabbit erythrocytes was found in gel filtration fractions containing such fragments: any reaction was not observed either in the direct agglutination test or in a modified antiglobulin test.

Key words: Immunoglobulin-fragments; copro-antibodies.

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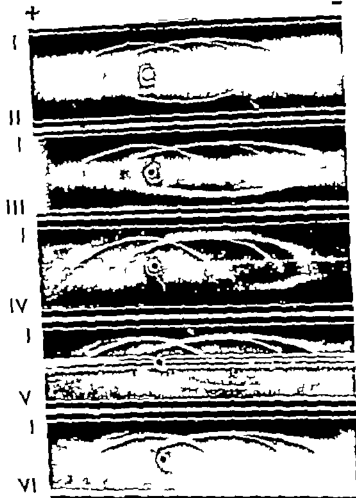
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IgA is found in extracts of faeces from most infants and children, while IgG and IgM are only demonstrable in a small percentage (6). The concentrations of IgA, in relation to dry weight of faeces, exceed those of the other classes. This difference can, to some extent, be explained by a preponderance of IgA-containing plasma cells in the intestinal mucosa (2) indicating a high level of production. In addition, the relative resistance of high-molecular-weight secretory IgA to proteolytic enzymes (11) may contribute to the dominance of IgA in faeces since the number of intestinal plasma cells containing IgA is not equally marked in relation to the numbers of cells containing IgG and IgM (2). A large part of the IgG and IgM found in the upper intestinal tract (1, 10, 12) is probably more or less degraded during passage through the gut. If exposed to pepsin at low pH even secretory IgA will be fragmented (11), and large amounts of IgA subjected to this process are found in gastric secretion (8). A proteolytic enzyme probably derived from enteric microorganisms may also split secretory IgA (9). Normally, however, only IgA in faeces seems to possess antibody activity if tested against rabbit erythrocytes (5): this is likely to be the result of a different sensitivity of the immunoglobulins to the intestinal enzymes.

Since fragments of immunoglobulins can interfere with the quantitation of immuno-

Hotchin J & *Cinits M* Lymphocytic choriomeningitus infection of mice as a model for the study of latent virus infection. Canad. J Microbiol *4* 149–163 1958
Hotchin J E. & *Weigand H* Studies on lymphocytic choriomeningitus in mice. J Immunol *86* 392–400 1961
Hotchin J & *Weigand H* The effect of pretreatment with x rays on the pathogenesis of lymphocytic choriomeningitis in mice I Host survival virus multiplication and leukocytosis. J Immunol. *87* 675–681 1961
Kärber G Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Naunyn-Smiedebergs Arch. Exptl. Pathol. Pharmakol. *162* 481–483 1931
Lundstedt C LCM virus, pathogenetic studies. Thesis. FADLs forlag Kobenhavn, Århus Odense 1974 *p 52*
Marker O & *Volkert M* Studies on cell mediated immunity to lymphocytic choriomeningitis virus in mice J Exp Med *137* 1511–1525 1973
Marker O & *Volkert M* In vitro measurement of the time course of cellular immunity to LCM virus in mice *Editor F Lehmann* Grube Springer Verlag Berlin Heidelberg New York 1973 p 207–216
Mims C A & *Blanden R V* Antiviral action of immune lymphocytes in mice infected with lymphocytic choriomeningitis virus. Infect. & Immunity 6 695–698 1972.
16 *Mims C A* & *Tosolini F A* Pathogenesis of lesions in lymphoid tissue of mice infected with lymphocytic choriomeningitis (LCM) virus. Br J exp Path *50* 584 592 1969.
17 *Rowe W P* Protective effect of pre-irradiation on lymphocytic choriomeningitis infection in mice Proc. Soc. Exp Biol., N Y 92 194 *198 1956*
18. *Rowe W P* Studies on pathogenesis and immunity in lymphocytic choriomeningitis infection of the mouse. Nav Med. Res. Inst. Rep *12* 167–220 1954
19 *Traub E* Factors influencing the persistence of LCM virus in the blood of mice after clinical recovery J Exp. Med. *68* 229-230, 1938.
20 *Volkert M* Studies on immunological tolerance to LCM virus. Acta path. microbiol scand *56* 305-310 1962.
21 *Volkert M* *Marker O* & *Bro-Jorgensen K* Two populations of T lymphocytes immune to the lymphocytic choriomeningitis virus. J Exp Med *139* 1329–1343 1974
22 *Zinkernagel R M* & *Doherty P C.* Cytotoxic thymus-derived lymphocytes in cerebrospinal fluid of mice with lymphocytic choriomeningitis. J Exp. Med. *138* 1266-1269, 1973

Fig. 1. Immunoelectrophoresis of normal faecal extract against antiserum to I) whole human serum, II) IgA, III) IgG (Fab) IV) IgG (Fd) V) kappa light chains and VI) IgG. An additional weak precipitation line produced by the antiserum to IgA is indicated by the arrow.



eluted in the same region as α1 antitrypsin and albumin (Fig. 2). Hence, the components were likely to represent monovalent Fab-fragments and not F(ab')$_2$. The antiserum to IgG (Fab) gave a strong precipitate in double diffusion with extracts of faeces from all of 100 healthy infants and children. Only a faint precipitate between antisera to IgG or IgG (Fc) and 8 of these individual extracts was seen. Thus, Fab-fragments of IgG seemed to be present in faeces from normal individuals.

The faecal IgG (Fab) precipitate was also seen in patients with extremely low serum IgA and no demonstrable IgA in faeces. Faecal extracts from 3 patients with agammaglobulinaemia had no γ-precipitates on immunoelectrophoresis. In double diffusion, however, a weak reaction with the extract from one of them against antiserum to IgG (Fab) was observed.

The gel filtration fractions likely to contain Fab-fragments of IgG did not agglutinate rabbit erythrocytes. Antiglobulin tests of extracts of faeces and gel filtration fractions, using antiserum to IgG (Fab) did not increase the titre or give visible agglutination of rabbit erythrocytes. It therefore seems as if the Fab-fragments of IgG in faeces fail to react with these erythrocytes.

Immunoelectrophoresis of the extracts of faeces from 2 of the healthy individuals re-

globulins (11) we investigated their presence in the faecal extracts used. In this paper the identification of the fragments normally present in faeces using conventional antisera and separation techniques, are described. The fragments were also tested for activity against rabbit erythrocytes.

MATERIALS AND METHODS

Individuals. One hundred healthy infants and children, 1 month to 12 years of age, were randomly selected at well baby clinics and among children of hospital employees. None of the babies were receiving human milk. Three patients with ulcerative colitis in clinical remission and with high faecal levels of IgG, 2 patients with IgA-deficiency and 3 patients with agammaglobulinaemia (6) were also included.

Faecal extracts. Samples of faeces, free from blood, were collected and extracts were made using 10 ml phosphate buffered saline (PBS) pH 7.2 per g freeze-dried faecal material as described previously (7).

Sera. Antisera to whole human serum, IgA, IgG and IgM were obtained by immunization of rabbits and made specific for heavy chains by absorptions with rabbit erythrocytes agglutinated by different immunoglobulin classes (7). The corresponding antisera as well as rabbit antisera to human IgG (Fab), IgG (Fc)*, IgG (Fd)*, light chains of kappa and lambda types and to free light chains (Bence-Jones) of both types, were purchased (Behringwerke AG, Marburg Lahn, Germany). A pool of sera from 10 healthy blood donors served as normal human serum. Gammaglobulin 16.5 per cent (Kabi, Stockholm, Sweden) was used as a source of human IgG.

Gel filtration. Sephadex G-200 column 1.5 × 45 cm, equilibrated at pH 8 with 0.05 M tris HCl buffer with 0.14 M NaCl were used for separation of proteins in 10 different concentrated faecal extracts as outlined earlier (7). Normal human serum was run as control for elution regions of the various proteins.

Agglutination test. Gel filtration fractions were tested for agglutinins to rabbit erythrocytes as previously described (5).

Antiglobulin test. Two-fold dilutions of faecal extracts or gel filtration fractions of faecal extracts were used for sensitization of rabbit erythrocytes and tested for agglutination with antiserum to IgG (Fab) and not as previously (5) with antiserum to IgG.

Polyacrylamide gel electrophoresis. The precipitates obtained from one faecal extract mixed with antiserum to IgA were repeatedly washed in large volumes of PBS and dissolved in buffer containing either 8 M urea or 1 per cent sodium dodecyl sulphate before electrophoresis in 7.5 per cent polyacrylamide gel with urea (3) and in 7.5 and 15 per cent gel with dodecyl sulphate (15). Precipitates prepared from normal human serum with antiserum to IgA were used as control.

Other methods. Double diffusion and immunoelectrophoresis in agar were carried out as formerly described (7). Fragmentation of IgG by trypsin (Sigma Chemical Corp., St. Louis, Missouri, U.S.A.) 1 per cent w/w was performed as outlined by *Waller et al.* (14).

EXPERIMENTS AND RESULTS

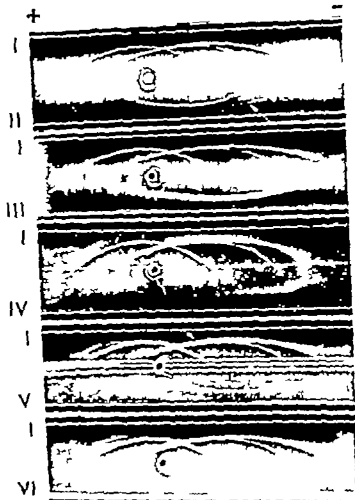
Immunoelectrophoresis of extracts of faeces from 12 healthy individuals, using antisera to whole human serum, invariably showed a strong precipitation line in the γ-region in addition to the IgA line and 1 or 2 lines in the α region (Fig. 1 I). The mobility of the marked γ-line was intermediate between those of serum IgA and IgG. A reaction of partial identity with this precipitation line and those obtained with antisera to IgG (Fab), IgG (Fd) and to light chains of both kappa and lambda types was observed (Fig. 1 III, IV, V). However, no precipitate was formed with antisera to either IgG (Fig. 1 VI) or IgM, nor with antisera to IgG (Fc) or free light chains (Bence Jones). The antisera to IgG (Fab) or IgG (Fd) gave no precipitate in double diffusion against purified IgA, and the strong precipitation reaction with faecal extracts was not inhibited by absorption of the antisera with rabbit erythrocytes agglutinated by IgA or IgM. Thus, the precipitate did not solely represent light chains.

In a control system with trypsin treated IgG, the antisera to IgG reacted with both Fab- and Fc-components, while the antiserum to whole serum reacted mainly with the Fab-component as identified by immunoelectrophoresis. Here too, absorption of the antisera with other classes of immunoglobulins, to remove anti light-chain activity, did not influence the reactions.

Gel filtration through a Sephadex G 200 column indicated that the components reacting with the antiserum to IgG (Fab) were

* Manufacturer's terminology

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The results of gel filtration experiments indicate that the molecular size of any fragments of IgA present was close to that of Fab-fragments of IgG α antitrypsin and albumin. The multiple bands obtained by polyacrylamide gel electrophoresis in urea also suggests that it is not only intact IgA in faeces which is precipitated by the antiserum to IgA. Dodecyl sulphate was used in order to obtain information about the size of the molecules reacting with the antiserum to IgA. The results, like those obtained in urea-electrophoresis, were not unequivocal since the IgA_2 subclass lacks the disulphide bridges between the heavy and light chains, and therefore will probably be fragmented during analysis (4). However only one or two "small-molecular-bands representing light chains are then reported to be formed (4). In our experiments, α-heavy chain determinants were demonstrated in all the regions of the urea gel corresponding to stained bands. The serum control appeared to confirm that the smaller molecules to be observed when this technique is used were not solely caused by in vitro fragmentation of IgA although this subclass makes up a relatively small part of the IgA in serum compared to secretions (4 13).

Fragments of IgA may interfere with the quantitative determination of IgA in single radial immunodiffusion smaller molecules diffuse more readily through agar resulting in erroneously high concentrations (11). In our system, however the late appearance and the weak precipitation of the presumed fragments of IgA seemed to exclude the possibility of any such influence on the results of quantitation. The fragments found may explain the multiple precipitin rings observed by others (10).

The findings in extracts of faeces from patients with immunoglobulin deficiencies seemed to confirm the specificity of the reactions observed. The presence of fragments of IgG appeared to be independent of the presence of fragments of IgA. Fragments of IgA were not seen in faecal extracts from IgA deficient patients.

In IgA-deficient individuals, the relatively large amounts of IgM excreted with faeces retain some antibody activity against rabbit erythrocytes (6). However no antibody activity of faecal IgG was demonstrated, even when present in faeces in amounts corresponding to those of IgM. Neither did the antiserum to IgG (Fab) reacting strongly with the presumptively large amounts of fragmented IgG lead to agglutination of the rabbit erythrocytes in the antiglobulin test. It therefore seems probable that the activity of IgG in the gut is shortlived.

REFERENCES

1. Bull, D. M. *Bienenstock J. & Tomasi T. B. jr.* Studies on human intestinal immunoglobulin A. Gastroenterology 60 570–580 1971
2. *Crabbé P. A. & Heremans J. F.* The distribution of immunoglobulin-containing cells along the human gastrointestinal tract. Gastroenterology *51* 305–316, 1966.
3. *Davis B. J.* Disc electrophoresis. II Method and application to human serum proteins. Ann. N.Y. Acad. Sci. *121* 404–427 1964
4. *Grey H. M., Abel, C. A. Yount W. J. & Kunkel H. G.* A subclass of human γA-globulins (γA_2) which lacks the disulphide bond linking heavy and light chains. J. exptl. Med. *128* 1223–1236 1968.
5. *Hanseberg B.* Human fecal agglutinins to rabbit erythrocytes. Scand. J. Immunol. 3 71 76, 1974

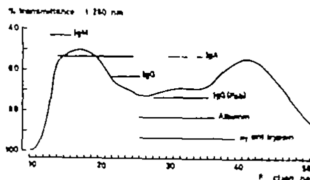


Fig 2 Gel filtration through Sephadex G-200 (1.5 × 45 cm) of a ten fold concentrate of normal faecal extract The flow rate was 11 ml/cm²/hour and fractions, about 2 ml each were collected during continuous registration of UV light transmittance The presence of precipitates in double diffusion of the fractions and various antisera is indicated The dashed line indicates a faint precipitate

vealed a double precipitation line with antisera to IgA (Fig 1 II) One of the lines gave a reaction of identity with the IgA line against antisera to whole human serum. It is possible that the other line (indicated by arrow) represents fragments of IgA, as demonstrated for IgG The results of Sephadex G 200 gel filtration support this fractions from the region in which the fragments of IgG were found, gave a precipitate in double diffusion and in single radial immunodiffusion with antisera to IgA (Fig 2) This precipitate was distinctly weaker than the usual IgA precipitate being visible only after at least 48 hours incubation Moreover absorptions of the antisera to IgA with other immunoglobulin classes did not remove this precipitate which was not found in the gel filtration fractions of faecal extracts from patients with IgA-deficiency and thus, it was not cross-reacting with Fab-fragments of IgG

Polyacrylamide gel electrophoresis of precipitates from faecal extract obtained by antiserum to IgA and dissolved in urea, gave at least 4 bands distinct from the intact immunoglobulin (Fig 3) The results of parallel controls using normal human serum precipitated by the same antiserum to IgA gave no bands below the usual immunoglobulin level. After electrophoresis in urea slices of an unstained gel, corresponding in position to the stained bands, were put into a small amount of PBS dialysed and freeze-dried before they were dissolved in 100 µl PBS. These extracts of the gel all gave a precipitate in agar against antiserum to IgA. Electrophoresis using sodium dodecyl sulphate gave at least 3 bands, indicating the presence of fragments with molecular weights 50,000-100 000 (Fig 3)

DISCUSSION

The present results of immunoelectrophoresis, double diffusion and gel filtration provide evidence for the occurrence of Fab-fragments of IgG in extracts of faeces from healthy infants and children. The absence of reactivity with antisera to γ-heavy chains may be the result of proteolytic action on intestinal IgG which is probably more pronounced in the gut than in the control system in which trypsin is acting on IgG *in vitro* Results of this *in vitro* degradation indicated that the antisera to γ-heavy chains are capable of reacting with the Fab- as well as the Fc frag-

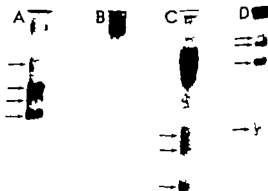


Fig 3 Polyacrylamide gel electrophoresis in 8 M urea (A, B) and in 1 per cent sodium dodecyl sulphate (C D) of precipitates of faecal extract (A C D) and of normal human serum (B) obtained using an antiserum to IgA. The urea gel was 7.5 per cent and the dodecyl sulphate gel 7.5 per cent (C) and 15 per cent (D) Stained bands below the band corresponding to intact immunoglobulins are indicated by arrows.

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REFERENCES

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2. *Crabbé P A & Heremans J F*. The distribution of immunoglobulin-containing cells along the human gastrointestinal tract. Gastroenterology *51* 305–316 1966.
3. *Davis B J* Disc electrophoresis. II Method and application to human serum proteins Ann. N Y Acad. Sci. *121* 404–427 1964
4. *Grey H M., Abel, C A Yount, W J & Kunkel H G* A subclass of human γA-globulins (γA_2) which lacks the disulphide bonds linking heavy and light chains. J exptl. Med. *128* 1223–1236 1968.
5. *Hammarberg B.* Human fecal agglutinins to rabbit erythrocytes. Scand. J Immunol. *3* 71 76, 1974

Haneberg B & *Aarskog D* Human fecal immunoglobulins in healthy infants and chil dren, and in some with diseases affecting the intestinal tract or the immune system Clin. exp Immunol In press 1975

Haneberg B & *Tønder O* Immunoglobulins and other serum proteins in feces from infants and children Scand J Immunol *2* 375–383 1973

McClelland D B L. Finlayson N D C., Samson R R Nairn I M & *Shearman D J C* Quantitation of immunoglobulins in gastric juice by electroimmunodiffusion. Gastroenterology *60* 509–514 1971

Mehta S K Plaut A G Calvanico N J & *Tomasi T B jr* Human immunoglobulin A Production of an Fc fragment by an enteric microbial proteolytic enzyme. J Immunol. *111* 1274–1276 1973

Plaut A G & *Keonil P* Immunoglobulins in human small intestinal fluid Gastroenterology *56* 522–530 1969

11 *Samson R R McClelland D B L* & *Shearman D J C* Studies on the quantitation of immunoglobulins in human intestinal secretions. Gut *14* 616–626, 1973

12. *Savilahti E.* Immunoglobulin-containing cells in the intestinal mucosa and immunoglobulins in the intestinal juice in children. Clin. exp. Immunol *11* 415–425 1972.

13 *Vaerman J P* Subdivision of IgA in two subclasses. In Studies on IgA immunoglobulins in man and animals, Louvain, 1970, p 69–86

14 *Waller M Richard A J* & *Mallory J* Immunochemical and serological studies of enzymatically fragmented human IgG globulins II Hydrolysis with subtilisin, elastase trypsin and chymotrypsin. Immunochemistry *6* 207 214 1969

15 *Weber K* & *Osborn M* The reliability of molecular weight determinations by dodecyl sulphate-polyacrylamide gel electrophoresis J Biol Chem. *244* 4406–4412 1969

Acta path. microbiol. scand. Sect. C, 84: 37-44, 1976

THE EFFECT OF PHENYLBUTAZONE AND CHLORAMPHENICOL ON PHAGOCYTOSIS OF RADIOLABELLED *CANDIDA ALBICANS* BY HUMAN MONOCYTES CULTURED *IN VITRO*

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Ødegaard, A. & Lamvik, J. The effect of phenylbutazone and chloramphenicol on phagocytosis of radiolabelled *Candida albicans* by human monocytes cultured *in vitro*. Acta path. microbiol. scand. Sect. C, 84: 37-44, 1976.

The effect of phenylbutazone and chloramphenicol on the function of blood monocytes cultured *in vitro* was studied. Both drugs had an inhibitory effect on the engulfment stage of phagocytosis. While moderate effect only of chloramphenicol on digestion of engulfed yeast particles was found, phenylbutazone caused a marked reduction of the digestion of engulfed particles. Concomitant with this reduction of digestive ability the lysosomes showed no morphological alterations as observed in control cultures without drug addition, indicating a lack of fusion between the engulfed particles and lysosomes in the presence of phenylbutazone.

Key words: Mononuclear blood cells; phagocytosis; effect of phenylbutazone and chloramphenicol.

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Phenylbutazone, an anti-inflammatory drug, has for years occupied an important place beside the salicylates and corticosteroids in the treatment of rheumatic diseases.

Phenylbutazone does not influence the course of rheumatic disease, but it decreases the host reaction thus preventing excessive inflammatory response which is injurious. There are indications that phagocytosis and the subsequent release of lysosomal enzymes play a central role in the inflammatory response. Phenylbutazone may exert its effect by stabilizing lysosomes against a wide variety of injurious agents (11).

Human monocytes improve their phagocytic abilities if cultured *in vitro* (15) apparently along with an increase in their numbers of lysosomes.

The present study deals with the effect of phenylbutazone and of chloramphenicol, an inhibitor of protein synthesis, on engulfment and intracellular digestion of radiolabelled Candida particles by human monocytes, using standardized techniques for the registration of phagocytosis and cell survival.

MATERIALS AND METHODS

The methods for culturing of human monocytes and for registration of drug effect on different functions of mononuclear blood cells have been described in detail previously (12, 13). Registra-

tion of cellular adhesiveness and survival of mononuclear adhesive cells after exposure to the drugs was performed as described as test model I in a previous report (12)

Phenylbutazone (Ciba-Geigy) was dissolved in 1 N NaOH and diluted with sterile distilled water Chloramphenicol succinate (Dumex) was dissolved in and diluted with sterile distilled water

Monocytes cultured on coverslips were tested for phagocytosis after 8 days culturing The effect of phenylbutazone and chloramphenicol on attachment/engulfment and on digestion of heat killed Candida particles radiolabelled with [1] [5]Iodine (8) was studied in separate experiments and registered according to the methods described previously as *test model II (12)*

In the attachment/engulfment experiments, the cells were incubated in the presence of a constant number of Candida particles and the drugs used in varying concentrations. Drug and Candida particles were added simultaneously The cells were harvested after a period of phagocytosis of 10 minutes In the attachment/engulfment and digestion experiments *the ratio between the number of* Candida particles added and the number of adhesive cells was usually about 20 1 In the digestion experiments, a constant number of Candida was added to coverslip cultures. After further incubation *for* 10 minutes the cells were washed three times in Hanks balanced salt solution (BSS) and placed in new Petri dishes containing fresh culture medium. Phenylbutazone or chloramphenicol in varying concentrations were added to the culture medium one hour later The cultures were harvested after incubation for a further 23 hours. The radioactivity in the supernatant and in the sediment following high speed centrifugation (2000 *g* in 10 min) was measured separately The radioactivity on the coverslips was registered as a measure of the amount of *Candida left in the cells* on the coverslips.

In the digestion experiments, at the time of harvesting, some of the coverslips were rinsed in BSS and inverted on microchambers as described previously (13) The chambers were filled with Parkers medium 199 supplemented with 20 per cent homologous AB serum and studied in a Leitz Laborlux phase contrast microscope with automatic equipment for photography

RESULTS

Cellular Adhesiveness

The effect of phenylbutazone on the number of *leucocytes which adhered to the bot*tom of the plastic Petri dishes after an incubation period of 90 minutes is shown in Table 1 The numbers of adhesive cells were estimated as the difference between the numbers of leucocytes added to the culture dishes and the numbers of cells removed after incubation for 90 minutes. Phenylbutazone in a final concentration ranging between 0.5 mg/ml and 6 mg/ml slightly reduced the adhesiveness of the mononuclear blood cells. No definite effect of *chloramphenicol on the* attachment of mononuclear leucocytes was observed Ninety to 95 per cent of the adhesive cells were phagocytic as judged by their ability to adhere Candida particles.

Cell Survival

Tables 2 and 3 show the survival of the adherent cells, estimated on day 4 of culturing when the cells had been incubated with

TABLE 1 *Effect of Phenylbutazone on the Adhesiveness of Blood Mononuclear Cells*

Culture series no.	Cell numbers × 10⁶ added	Numbers of adhesive cells after exposure to phenylbutazone in the following concentration (mg ml)					
		0	0 5	1	2	4	6
66	7 5	2 4	2 0	2 0	1 9		1 1
67	7 5	1 0	1 0	0 5	0 2	0 3	–
68	7 5	2 4	1 0	2 1	1 2	1 5	1 4
98	7 5	3.2	3.5	2 7	1 9	1 9	2 0
128	7 5	2 3	1 9	–	2 3		1 9
131	7 5	1 2	1 0	0 9	1 2	0 3	0 4

Numbers of adhesive cells per culture dish × 10⁶ estimated as the difference between the numbers of cells added to the culture dishes and the numbers *of* cells removed *after 90 minutes incubation.*

– not tested

TABLE 2. *Effect of Phenylbutazone on Survival of Blood Mononuclear Phagocytes Cultured* In Vitro

Culture series no.	Numbers of surviving cells after exposure to phenylbutazone in the following concentrations (mg/ml)					
	0	0.5	1	2	4	6
66	11.5	7.0	9 1	0	0	0
67	1.9	1.3	1.3	0	0	0
68	13 9	–	12 6	0	0	0
98	3.2	2.5	2 1	0	0	0
128	3.9	–	–	0	0	0
131	1.9	0 4	0	0	0	0

The cells were exposed to the drug for 90 minutes at culture start and the numbers of adhesive phagocytes counted after 4 days culturing. The values given are the means of the cell numbers $\times$ 10[4] in triplicate culture dishes
– not tested.

TABLE 3 *Effect of Chloramphenicol on Survival of Blood Mononuclear Phagocytes Cultured* In Vitro

Culture series no.	Numbers of surviving cells after exposure to chloramphenicol in the following concentrations (mg/ml)					
	0	1.25	2.5	5	10	20
171	9.5	–	9 7	8.5	5.7	0
172	33.5	–	–	32.0	13.2	0
173	19.6	19 4	20.9	23.9	20 1	0
174	17 9	13 0	18 6	13 6	15.0	0
175	14 0	15 4	18.1	16.8	12.5	0
176	50 3	48.5	46 7	33.0	23.0	0

For explanation of the figure see Table 2
– not tested.

various concentrations of the drugs at culture start. A marked inhibitory effect on cell survival was found when the cells had been exposed to more than 1 mg/ml of phenylbutazone or to more than 10 mg/ml of chloramphenicol.

Attachment/Engulfment of Radiolabelled Heat Killed Candida

Phenylbutazone in concentrations exceeding 2 mg/ml and chloramphenicol in concentrations higher than 5 mg/ml decreased the attachment/engulfment to 8 days old human mononuclear phagocytes (Table 4 and 5).

Digestion of Radiolabelled Heat Killed Candida

Phenylbutazone in final concentrations ranging between 0.5 mg/ml and 6 mg/ml decreased the release of [125]Iodine to cell free medium, indicating a reduced intracellular digestion of the engulfed particles (Table 6, Fig. 1). The cells were observed microscopically at harvesting (Fig. 3). At drug concentrations between 2 mg/ml and 6 mg/ml, many of the cells were detached from the coverslips as measured by the amounts of radioactivity in the sediment (Fig. 1). The remaining cells showed many undigested particles left in the cells without any morphological alteration of the lysosomes (Fig. 3). A moderate effect only of chloramphenicol on

TABLE 4 *Effect of Various Concentrations of Phenylbutazone on the Attachment/Engulfment of Radio-labelled Heat Killed* Candida albicans *in 8 Days Old Blood Mononuclear Phagocytes Cultured* in Vitro

Culture series no.	Radioactivity in coverslip cultures exposed to phenylbutazone in the following concentrations (mg/ml)					
	0	0.5	1	2	4	6
134	28.8	29.5	37.3	25 9	18 1	13.5
135	41 7	40 6	49.5	31.2	26 7	21 9
136	38.2	–	38.2	41 4	32 9	19.9
137	36.5	35 8	34 4	31 8	26.5	19 7
138	39.5	38 7	33.5	32.8	27.5	21.5

The figures listed are the means of the results (ct/min $\times$ 10^3) in quadruplicate coverslips.
– not tested.

TABLE 5 *Effect of Various Concentrations of Chloramphenicol on the Attachment/Engulfment of Radio-labelled Heat Killed* Candida albicans *in 8 Days Old Blood Mononuclear Phagocytes Cultured* in Vitro

Culture series no	Radioactivity in coverslip cultures exposed to chloramphenicol in the following concentrations (mg/ml)					
	0	1.25	2.5	5 0	10 0	20.0
160	92.3	78.2	83.5	71 6	–	36.5
161	90 4	78 6	63 6	62.0	50 0	32.9
170	32.0	26 6	–	27 4	22.4	14.2
180	93.9	91 1	86.9	86.6	89.2	62.2
181	66.8	65 0	61 7	66 1	33 4	50 7
183	38.5	34 3	36 9	23 6	12 1	12.2

For explanation of the figure see Table 4
– not tested

TABLE 6 *Effect of Various Concentrations of Phenylbutazone on the Digestion of Engulfed Radio-labelled Heat Killed* Candida albicans *in 8 Days Old Blood Mononuclear Phagocytes Cultured* in Vitro

Part of culture system	Radioactivity in different parts of the culture system after exposure to phenylbutazone in the following concentrations (mg/ml)					
	0	0 5	1	2	4	6
coverslips	27 15 11 1	18 18 11–15	12 13 15 11	16 7 14 14	21 18 20 31	21 21 35 34
sediment	11 13	15 13	16 20	57 57	54 41	33 78
cell free medium	164 125	142 109	102 118	90 78	65 70	51 55
total per culture dish	211 164	192 148	144 164	170 179	157 164	126 152

The figures listed are the results (ct/min $\times$ 10^3) from one characteristic digestion experiment performed in duplicate culture dishes each dish containing duplicate coverslips. Radioactivities in different parts of the culture system are given.

TABLE 7 *Effect of Various Concentrations of Chloramphenicol on the Digestion of Engulfed Radio-labelled Heat-Killed* Candida albicans *in 8 Days Old Blood Mononuclear Phagocytes Cultured in Vitro*

Part of culture system	Radioactivity in different parts of the culture system after exposure to chloramphenicol in the following concentrations (mg ml)					
	0	1.25	2.5	5	10	20
coverslips	27 22 27 25	18-30 33-29	31 23 25-38	29-40	28-30 25-39	10 - -
sediment	13 15	10 19	13 11	27 -	29 25	177 90
cell free medium	73 67	60 78	64 67	60 -	63 60	41 35
total per culture dish	133 134	118 159	130 141	156 -	150 149	185 139

For further explanation of the figures see Table 6.
not tested.

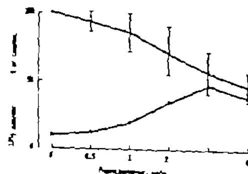


Fig. 1 Effect of phenylbutazone on digestion of [125]I-labelled heat-killed Candida particles in blood mononuclear phagocytes cultured for 8 days on coverslips. The values are the means of the results obtained in 6 experiments performed in duplicate culture dishes and registered 23 hours following the engulfment phase. Results are expressed as percentages of the radioactivity in the medium in control cultures without drug addition.
● Release of [125]I-radioactivity to cell free medium.
▲ [125]I-radioactivity in sediment.

the digestive ability of the mononuclear cells was observed in concentrations from 1 to 10 mg ml (Table 7 Fig. 2)

DISCUSSION

Two effects of phenylbutazone on human blood monocytes cultured *in vitro* have been observed. Firstly phenylbutazone in concentrations higher than 2 mg/ml damaged the adhesive cells to such a degree that very few survived for four days in culture. The effect on cellular adhesiveness was only slight. Secondly although a moderate effect on the attachment/engulfment stage of phagocytosis was observed, a very marked effect on intra

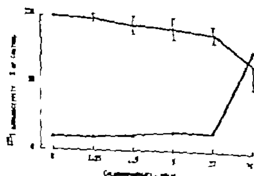


Fig. 2 Effect of chloramphenicol on digestion of [125]I-labelled heat-killed Candida particles in blood mononuclear phagocytes cultured for 8 days on coverslips. The values are the means of the results obtained in 6 experiments performed in duplicate culture dishes and registered 23 hours following the engulfment phase. Results are expressed as percentages of the radioactivity in the medium in control cultures without drug addition.
● Release of [125]I-radioactivity to cell free medium.
▲ [125]I-radioactivity in sediment.

TABLE 4 *Effect of Various Concentrations of Phenylbutazone on the Attachment/Engulfment of Radio-labelled Heat Killed* Candida albicans *in 8 Days Old Blood Mononuclear Phagocytes Cultured* in Vitro

Culture series no.	Radioactivity in coverslip cultures exposed to phenylbutazone in the following concentrations (mg/ml)					
	0	0.5	1	2	4	6
134	28 8	29..	37.3	25 9	18.1	15.5
135	41 7	40 6	49.5	31.2	26 7	21.9
136	38.2	–	38.2	41 4	32.9	19.9
137	36.5	35.8	34 4	31.8	26.5	19 7
138	39.5	38 7	33.5	32.8	27.5	21.5

The figures listed are the means of the results (ct/min × 10²) in quadruplicate coverslips.
– *not tested*

TABLE 5 *Effect of Various Concentrations of Chloramphenicol on the Attachment/Engulfment of Radio-labelled Heat Killed* Candida albicans *in 8 Days Old Blood Mononuclear Phagocytes Cultured* in Vitro

Culture series no.	Radioactivity in coverslip cultures exposed to chloramphenicol in the following concentrations (mg/ml)					
	0	1 25	2.5	5 0	10 0	20.0
160	92.3	78.2	83.3	71 6	–	36.5
161	90 4	78 6	63 6	62.0	30 0	32.9
170	32 0	26.6	–	27 4	22.4	14.2
180	93 9	91 1	86 9	86 6	89.2	62.2
181	66 8	65 0	61 7	66 1	53 4	50 7
183	38.5	34 3	36 9	23 6	12 1	12.2

For explanation of the figure see Table 4
– not tested

TABLE 6 *Effect of Various Concentrations of Phenylbutazone on the Digestion of Engulfed Radio-labelled Heat Killed* Candida albicans *in 8 Days Old Blood Mononuclear Phagocytes Cultured* in Vitro

Part of culture system	Radioactivity in different parts of the culture system after exposure to phenylbutazone in the following concentrations (mg/ml)					
	0	0.5	1	2	4	6
coverslips	22–15 11–12	*18 18* 11–15	12 13 13 11	16 7 14 14	21 18 20 33	21 21 35–34
sediment	11 13	15 13	16 20	57 57	54 41	33 28
cell free medium	164 125	142 109	102 118	90 78	65 70	51 55
total per culture dish	211 164	192 148	144 164	170 179	157 164	126 152

The figures listed are the results (ct/min × 10) from one characteristic digestion experiment performed in duplicate culture dishes each dish containing duplicate coverslips. *Radioactivities in different parts* of the culture system are given.

cellular digestion of engulfed Candida particles was found as measured by the release of radioactivity to the cell free medium. Concomitant with this marked reduction in digestive ability induced by the drug, no morphological alteration of lysosomes was found and the degranulation of cytoplasmic structures which normally follows the digestive stage of phagocytosis was lacking.

Chloramphenicol caused a reduction of phagocyte survival only if the cells had been exposed to high concentrations of the drug. The effect of chloramphenicol on the digestion of yeast particles was only slight, while the effect on the attachment/engulfment stage of phagocytosis was moderate.

There are several mechanisms by which a drug may affect lysosomes and thus interfere with intracellular killing and digestion. Firstly the drug may have a damaging effect directly upon the lysosomal membrane followed by a leakage to the surrounding cytoplasm. Such a mechanism is indicated by the observation that impaired ability of human macrophages to digest yeast particles was found concomitant with a marked cytolytic effect following exposure to lytic concentrations of chlorpromazine (12). Secondly the drug effect may be associated with an inhibition of the formation of hydrogen peroxide having bactericidal properties as shown by *Strauss et al.* (8) and *Koch* (4). This observation may indicate that the drug acts secondarily upon lysosomes via an inhibitory effect on mitochondrial function. Thirdly a specific inhibitory effect of the drug on specific lysosomal enzymes would be expected to inhibit lysosomal digestion. It is documented that phagocytosis of foreign material leads to the intracellular formation of a so called phagosome, it is the engulfed particle surrounded by an inverted part of the cell membrane (3). The intracellular digestion starts when the phagosome fuses with the lysosome, whereby lysosomal enzymes can act directly upon the particle.

The cell morphological changes observed in this study may suggest a fourth mechanism of drug action, namely a lack of fusion between the engulfed particles and lysosomes.

The present findings are in a accordance with those obtained by *Strauss et al.* (8), *Solberg* (7) and *Koch* (4) who found that phenylbutazone decreases or inhibits the intracellular killing of engulfed bacteria in human neutrophilic granulocytes.

A slightly depressive effect only of phenylbutazone on cell membrane functions was found, as observed when the blood monocytes adhered to the surface of the culture dishes and when yeast particles became attached to the surface of the cells, with subsequent engulfment. A similar, although more marked effect on cell membranes was found by *Chang* (2) and *Kvarstein & Stormorken* (5) who observed that phenylbutazone decreased the engulfment of particles in human neutrophilic granulocytes.

It is not likely that the effect of phenylbutazone on cell survival *in vitro* found in the present study can be explained by the drug effect on cell surface membranes giving detachment of the cells. Since lysosomal membranes appear to be stabilized, a deleterious effect of lysosomal enzymes on the cell cytoplasm is also an unlikely explanation of the damaging effect on the cell.

Both drugs tested in the present study interfered with phagocytosis of particles in human cells cultured *in vitro*. A comparison

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Fig. 3. Effect of phenylbutazone on digestion of heat-killed *Candida albicans* in blood mononuclear phagocytes during 23 hours exposure to the drug.

A. Phenylbutazone added in final concentration of 6 mg/ml. The cell cytoplasm contains undigested Candida particles located in the perinuclear region of the cells and no obvious alterations of lysosomes are visible.

B. Phenylbutazone added in final concentration of 4 mg/ml.

C. Phenylbutazone added in final concentration of 2 mg/ml.

D. Control without phenylbutazone. No intact Candida particles can be seen in the cells. The cytoplasm is of granular appearance and thread-like membrane projections are visible.

Phase contrast 1480 ×


A

B

C

D

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ISOLATION OF C1q-BINDING IMMUNE COMPLEXES BY AFFINITY CHROMATOGRAPHY AND DESORPTION WITH A DIAMINOALKYL COMPOUND

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Svehag, S.-E. & Burger, D. Isolation of C1q-binding immune complexes by affinity chromatography and desorption with a diaminoalkyl compound. Acta path. microbiol. scand. Sect. C, 84 45–52, 1976.

The applicability of affinity chromatography to the isolation of C1q-binding immune complexes (IC) in sera was explored. Purified human C1q was covalently coupled to agarose or adsorbed to IgG-agarose resins. Sera containing preformed virus-antibody complexes or rheumatoid arthritis (RA) sera were passed through the columns and C1q-bound IC, eluted off with 1,4-diaminobutane at mild basic conditions, were analyzed by immunodiffusion, crossed immunoelectrophoresis, gel filtration and electron microscopy. Under conditions of antibody treatment which caused almost 100 per cent inhibition of virus plaque formation, about 30 per cent of formed ³H-labelled equine arteritis virus-antibody complexes was bound specifically to and desorbed from C1q-IgG agarose columns. Studies with RA-sera indicated the presence of both IgM-IgG and intermediate size IgG C1q-binding complexes in 3 out of 5 tested seropositive sera. In two sera only intermediate size IC were demonstrable. The results obtained in these two IC model systems suggested that the described methods could be useful for isolation of C1q-binding IC in general.

Key words: Immune complexes; affinity chromatography; diaminoalkyl compound.

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Antigen-antibody complexes have been directly implicated in injuries particularly affecting renal, cardiovascular and synovial tissues (9–15). Results obtained in experimental models have suggested that it is primarily complexes formed in moderate antigen excess which are associated with disease (8). Although there is lacking information concerning the spectrum of antigens that may participate in immune complex (IC) formation of pathogenic importance, nuclear, viral, malarial and streptococcal antigens, in particular, have been implicated in IC glomerulonephritis (1–3). In attempts to establish with greater certainty whether IC pathogenesis is involved in a variety of clinical diseases several methods for IC detection have been proposed recently (3–8, 11–14, 16, 19–27, 29). Techniques for isolation of adequate quanti-

between non toxic concentrations of the two drugs may indicate that phenylbutazone primarily exerts its effect on lysosomal membranes thereby reducing intracellular digestion whereas chloramphenicol which inhibits protein synthesis (1 9) and thereby interferes with several cellular functions, is without any marked effect on any particular stage of phagocytosis.

In recent years increasing evidence has been gained that phagocytosis of agents capable of disrupting lysosomal membranes may liberate lysosomal enzymes which in turn lead to cell and tissue injury and provoke inflammation (11) Immune reactions may result in the labilization of lysosomes and release of intracellular enzymes. *Quie & Hirsch* (6) have shown that antibodies to purified leucocyte lysosomes can induce degranulation of leucocytes and the release of lytic enzymes into the cell sap bringing about rapid cell death

Some agents useful in the treatment of inflammatory diseases leading to tissue damage appear to stabilize lysosomes. Phenylbutazone has been shown to retard the release of lysosomal enzymes into the circulation following irradiation in guinea pigs (11) The present findings are in support with the phenomenon observed by others that phenylbutazone has a stabilizing effect on lysosomal membranes, thereby reducing the digestive capacities of phagocytising cells The biological effect of the drug may mainly be that it reduces cell and tissue damage caused by lysosomal enzymes.

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REFERENCES

1 *Ambrose C T & Coons A H*. Studies on antibody production VII Inhibitory effect of chloramphenicol on synthesis of antibody in tissue culture. J Exp Med. *117* 1075-1088, 1963

2 *Chang Y* Studies on phagocytosis. The effect of nonsteroidal antiinflammatory drugs on phagocytosis and on urate crystal-induced canine joint inflammation. J Pharmacol. Exp. Ther *183* 235 244 1972

3 *Hirsch J G* Cinemicrophotographic observations on granule lysis in polymorphonuclear leucocytes during phagocytosis. J Exp. Med. *116* 827–833 1962

4 *Koch C* Effect of sodium azide upon normal and pathological granulocyte function. Acta path. microbiol scand. Sect. B, *82* 136-142, 1974

5 *Kvarstein B & Stormorken H* Influence of Acetylsalicylic acid, Butazolidin Cholchichine Hydrocortisone Chlorpromazine and Imipramine on the phagocytosis of polysterende latex particles by human leucocytes. Bioch. Pharm. *20* 119–124 1971

6 *Quie P G & Hirsch J G* Antiserum to leucocyte lysosomes its cytotoxic, granolytic, and hemolytic activities. J Exp Med. *120* 149–160 1964

7 *Solberg C O* Evaluation of neutrophil granulocyte function Acta path. microbiol scand. Sect B *80* 559–563 1972

8. *Strauss R. R Paul B B & Sbarra A J* Effect of phenylbutazone on phagocytosis and intracellular killing by guineapig polymorphonuclear leucocytes. J Bacteriol. *96* 1982 1990 1968

9 *Viken K E* 125I-labelling of Candida albicans by electrolysis. Acta path. microbiol scand Sect. B, *82* 219–222, 1974

10 *Weissberger A S Daniel T & Hoffman A J* Inhibition of protein synthesis in mammalian cell-free systems by chloramphenicol. J Exp Med *120* 161–183 1964

11 *Weissmann G* Lysosomes and Joint Diseases. Arthritis rheum *9* 834 840 1966

12 *Ødegaard A* Functional alterations induced by Chlorpromazine in mononuclear blood cells cultured in vitro. Acta path. microbiol scand Sect. C, *83* 59–73 1975

13 *Ødegaard A Viken K E & Lamvik J* Structural and functional properties of blood monocytes cultures in vitro Acta path. microbiol scand Sect. B *82* 223 234 1974

and the LA reaction was recorded after 20 hours incubation at room temperature. One agglutination unit correspond to 200 ng purified C1q preparation. All C1q preparations were derived from RF-negative sera. RF activity was determined at 37 C by essentially the same procedure, but to eliminate possible contributions of C1 or C1q to the observed titers the sera or preparations were tested both unheated and heated at 56 C for 40 min. The heat treatment reduced the agglutinating capacity of C1q by about 90 per cent.

Immunodiffusion Immunoelectrophoresis and Crossed Electrophoresis Analyses

Double diffusion analyses were performed in 1 per cent agarose (Bio Rad) and 0.15 M NaCl buffered with 0.01 M phosphate, pH 7.2 (PBS). Immunoelectrophoresis was carried out in 1 per cent agarose and sodium diethylbarbiturate buffer μ 0.05, pH 8.2, containing 0.01 M EDTA. Crossed immunoelectrophoresis was performed in 1 per cent agarose and barbital buffer pH 8.6, μ 0.02 with 10V/cm for 50 min in first dimension and with 2V/cm for 20 hours in second dimension.

Electron Microscopy

A Philips EM 200 equipped with an anti-contamination device and 50 μ objective aperture was used. Calibration of the magnification was performed with carbon grating having 54,864 lines/inch (Ladd Research Industries, Inc., Burlington, Vt.). The preparations to be examined were negatively contrasted with 2 per cent sodium tungstosilicate or ammonium molybdate, pH 7.0, on a 400-mesh carbon coated copper grid. Pictures were taken at an initial magnification of 46,600 at 60 kV and in through focal series.

Propagation and Titration of Equine Arteritis Virus

A stock of the Bucyrus strain of equine arteritis virus (EAV) was prepared in monolayer cultures of the NBL-6 cell line using Eagle's MEM with 10 per cent bovine serum as growth medium. EAV infectivity was determined by a plaque assay in NBL-6 cells (21).

Labelling of EAV with ¹⁴C-uridine

Monolayers of NBL-6 cells were infected with an input multiplicity of 20 PFU/cell. After 1 h at 37 C the monolayers were washed and covered with maintenance medium containing 20 μCi ¹⁴C-uridine (sp. act. 57 mCi/mM, New England Nuclear) and 0.5 μg/ml actinomycin D. Forty h after infection, the cells were exposed to three cycles of freezing and thawing. The cell debris was centrifuged down at low speed the virus was sedimented at 75,000 g for 8 h and the pellet was resuspended in PBS containing 20 per cent sucrose. Virus was purified further by density gradient centrifugation in a linear sucrose gradient (35–65 per cent) at 150,000 g for 10 h. The labelled and purified EAV was stored at −70° C.

Determination of Radioactivity in Effluent and Material Desorbed from C1q-IgG-Sepharose Resin

Effluent fluid and desorbed material were concentrated to the same extent, dialyzed against PBS, and to 1 ml of each was added 1 ml of ice-cold 5 per cent trichloracetic acid (TCA). The precipitate was collected on HOO paper filter in a suction flask and washed with 10 ml TCA, 10 ml absolute ethanol, 10 ml of an ethanol-chloroform-ether mixture, and finally with 10 ml ether. The filters were transferred to scintillation vials and dried overnight at 37 C. Five ml scintillation fluid consisting of toluene with 5 per cent butyl PBD (CIBA) were added and the vials were counted in a Philips scintillation spectrometer.

RESULTS

Detection and Isolation of C1q fixing Virus-antibody Complexes by Use of Affinity Chromatography

Purified and ¹⁴C-uridine labelled equine arteritis virus (EAV) was used as antigen and heat-inactivated equine immune serum as source of antibodies. Half a ml of immune serum and heat inactivated normal equine serum respectively were mixed with 1.5 ml labelled virus (10^7 PFU) and the mixtures were kept for 30 min at 37 C plus overnight at 4 C. Each sample was then applied to a separate C1q-IgG-Sepharose 6 B resin. After a reaction time of 40 min at room temperature the resins were washed exhaustively with 0.075 M Tris-HCl buffer pH 8.1 for 5 hours and bound material was desorbed with 0.2 M 1 4-diaminobutan in the same buffer.

Although the virus was neutralized to almost 100 per cent by the immune serum in plaque inhibition assays, up to 70 per cent of the labelled material passed through the C1q-IgG-Sepharose column (Table 1). The bound and desorbed material, containing the residual 30 per cent of the tracer was found to consist of horse IgG damaged virions and C1q when examined by immunodiffusion and

ties of IC permitting their subsequent characterization are however also desirable.

An important biologic property of many IC is their capacity to bind the C1q protein of the first component in the complement system. The feasibility of utilizing this property for the isolation of IC by affinity chromatography on C1q agarose columns is illustrated in the present study. Desorption of IC was attempted by the use of diaminoalkyl compound reported to inhibit the binding of IgG to C1q protein (6, 23, 30).

MATERIALS AND METHODS

Coupling of IgG to Sepharose 6 B

Human IgG (Cohn's fraction II, AB Kabi, Stockholm) was coupled to Sepharose 6 B (Pharmacia Fine Chemicals, Uppsala, Sweden) by the aid of cyanogen bromide (Kebo, Stockholm) using the method of *Cuatrecasas et al.* (7). To the activated Sepharose suspended in 40 ml chilled 0.1 M borate buffer, pH 9.0, was added 40 ml human IgG solution (5 mg/ml) and the coupling reaction took place at 4 C for 24 hours with slow stirring. The gel was washed with 200 ml chilled 0.1 M borate buffer, pH 9.0, followed by 100 ml 1 M glycin. 80 per cent of added IgG was bound to the Sepharose. The resin was washed with 500 ml 0.1 M acetate buffer, pH 4, containing 0.5 M NaCl and 500 ml 0.075 M Tris-HCl, pH 8.1, containing 0.075 M NaCl. 20 ml bed volume IgG-Sepharose columns were prepared and extensively washed with 0.075 M Tris-HCl, pH 11, containing 0.2 M 1,4-diaminobutan (Fluka AG, Buchs SG).

Coupling of C1q Protein to Sepharose 6 B

The procedure was essentially the same as that described above for IgG.

Preparation of C1q Protein

The C1q preparations were prepared from 100 ml fresh normal human serum as described by *Yonemasu & Stroud* (31). The final precipitate was dissolved in 5 ml of 0.75 M NaCl in 0.02 M acetate buffer and 0.01 M EDTA, pH 5.0. This C1q preparation was purified further on a Bio Rad A 15 m column (Fig. 1). The purity and activity of the final C1q preparations (2–3 mg/ml) was assayed by immunodiffusion, electron microscopy (24) and latex agglutination (LA) (10). The electron micrographs showed an abundance of the typical fan-shaped C1q molecules.

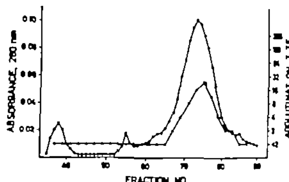


Fig. 1. Gel filtration on Bio Rad A 15 m of human C1q protein purified by euglobulin precipitation. Column size 1.9 × 180 cm. Sample 2 ml. The encircled fraction on the agglutination curve was chosen for purity examination by electron microscopy. Latex agglutination titer (○), absorbance at 280 nm (●).

Binding of C1q Protein to IgG-Sepharose Resins

Four ml (12 mg) of the C1q preparations (8 × 10⁴ LA units) were allowed to react for 1 hour at 4 C with the IgG-Sepharose resins and the columns were washed with 0.075 M Tris-HCl buffer for 20 hours.

Binding and Desorption of Immune Complexes

All sera were stored at −20 C prior to use. 0.5 to 2 ml serum was allowed to react with C1q-Sepharose or C1q-IgG-Sepharose resins for 40 min at room temperature. The columns were washed with 0.075 M Tris-HCl, pH 8.1, containing 0.075 M NaCl until the absorbancy had reached a low constant value. Bound material was desorbed with 0.075 M Tris-HCl, pH 11, containing 0.2 M 1,4-diaminobutan.

Gel Filtration

Sephadex G-200 or Bio Rad A 15 m columns, 1.4 × 85 cm or 1.9 × 180 cm in size, were equilibrated with 0.01 M Tris-HCl buffer containing 0.5 M NaCl and 0.02 per cent NaN_3, pH 8.0. The columns were kept at 4 C and the flow rate was 5 ml/hour. The protein contents in effluents was followed by T_{254} recording with Uvicord equipment or by E_{280} determinations in a Beckman DU spectrophotometer.

RA-latex Agglutination

C1q activity was determined by the method of *Ewald & Schubert* (10). Two-fold serial dilutions of purified human C1q protein were performed in physiological saline buffered with 0.05 M borate buffer, pH 8.2, in Takatsy plates and 25 µl of 1:5 diluted latex globulin reagent (Hyland RA-latex) was added to each well. The reagents were mixed

TABLE 2. *Immunodiffusion and Latex Agglutination Titration with Material Desorbed after Affinity Chromatography of Rheumatoid Arthritis Serum on C1q-Sepharose Resin and from Control Columns*

Column used	Immunodiffusion, desorbed material					Latex agglutination titre	
	IgG	IgM	IgA	Alb	C1q	wash fluid	desorbed material§
RA serum → C1q-Sepharose	+ + +	(+)	—	(+)	—	256	32
RA serum → IgG-Sepharose	+	+	—	—	—	32	128–256
Norm. serum → C1q-Sepharose	(+)	—	—	(+)	—	<2	<2

Peak fraction, same protein concentration.

§ Pooled fractions, same protein concentration.

passed through the C1q-Sepharose column, while the major RF activity was retained on IgG-Sepharose control resins (Table 2).

Results obtained with another serum (LA titer 4096) are illustrated in Figs. 2 b & 3 b. 0.5 ml serum was run on IgG-Sepharose and C1q-Sepharose columns respectively (Fig. 2 b). Desorbed materials were dialysed, concentrated 5× and 1.5 ml of each material was run consequtively on the same Bio Rad A 15 m column (Fig. 3 b). The material desorbed from the C1q-Sepharose column again contained high molecular weight IC and a substantial amount of intermediate complexes.

Material desorbed from the IgG-Sepharose resin appeared to contain primarily free IgM, compatible with its higher heat-stable LA titer (256 as compared to 32 in the material from the C1q-Sepharose resin).

Three of the RA-sera gave similar results, indicating the presence of both heavy and intermediate size complexes, as illustrated above. In the two additional sera tested, only C1q-binding IC of intermediate size were demonstrable.

Binding and Desorption of Rheumatoid Factor Using Sepharose-coupled Non-aggregated Human IgG and 1,4-diaminobutan

To examine further the material bound to IgG-Sepharose resins, 0.5 ml heat-inactivated RA serum (1024 LA units) was reacted with an IgG-Sepharose column and desorption was

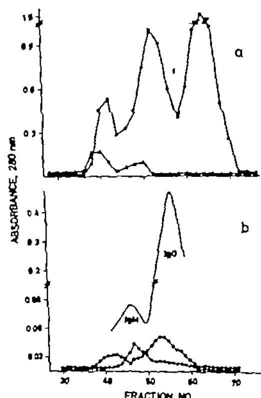


Fig. 3 a. Gel filtration on Sephadex G-200 of material (△) desorbed from a C1q-Sepharose 6 B column reacted with a rheumatoid arthritis serum (see Fig. 2). Reference run with normal human serum (○) on the same G-200 column.

Fig. 3 b. Gel filtration on Bio Rad A 15 m of the materials desorbed from a C1q-Sepharose (○) and an IgG Sepharose (●) column respectively (see Fig. 2 b) which had been reacted with the same rheumatoid arthritis serum.

TABLE 1 *Isolation of C1q-binding EAV-antibody Complexes by the Use of Affinity Chromatography and ^{14}C uridine Labelled EAV*

	Not bound tracer	Bound and desorbed tracer	Desorbed material		EAV neutralized
			IgG	EAV	
EAV and immune equine serum	70 %	30 %	+ +	+*	98 %
EAV and normal equine serum	99 %	1 %	–	–	0-5 %

* Damaged virions.

electron microscopy The C1q protein could be separated from the immune complexes on a subsequent G 200 column Control runs with EAV and normal equine sera on identically prepared C1q IgG-Sepharose columns showed only about 1 per cent binding of the labelled virus (Table 1)

Isolation of Immune Complexes from RA Sera by Use of C1q-Sepharose resin

Sera from five patients with classical rheumatoid arthritis (RA) (22) were used in this part of the study Fig 2 a illustrates the elution patterns obtained when one of these sera (LA titer 2.048) and a normal human serum (NHS) were run on two identically prepared C1q-Sepharose columns. Each column was reacted with 2 0 ml serum as described in Materials and Methods. Desorbed materials were dialysed concentrated and examined by immunodiffusion electron microscopy and crossed immunoelectrophoresis. IgG made up the major part of the protein desorbed from the C1q-Sepharose column reacted with RA serum but IgM and a small amount of albumin was also present (Table 2) 0.5 ml of the desorbed material was fractionated on a Sephadex G-200 column. The elution curve (Fig 3 a) suggested the presence of heavy (≥19S) IC and a lower yield of intermediate complexes. Crossed electrophoresis with rabbit anti IgM serum confirmed the presence of IgG IgM complexes in the heavy component and electron microscopy revealed aggregated material containing IgG and IgM but the aggregates were difficult to resolve Almost all heatstable LA activity (titer 256)

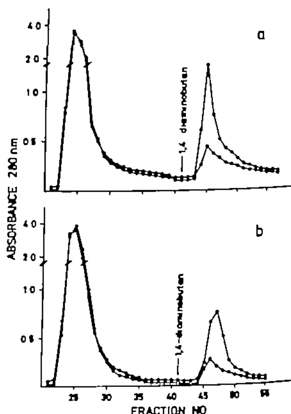


Fig 2 a Affinity chromatography of a rheumatoid arthritis serum (O) and a normal human serum (●) on two identically prepared C1q-Sepharose 6 B columns Each column was reacted with 2 ml serum for 40 min at room temperature, not bound material was eluted with Tris-HCl buffer pH 8.1 and retained material was desorbed with 0.2 M 1 4-diaminobutan in the same buffer

Fig 2 b Affinity chromatography of a rheumatoid arthritis serum on an IgG-Sepharose 6 B (●) and a C1q-Sepharose 6 B (O) column respectively Each column was reacted with 0 5 ml serum for 40 min at room temperature not bound material was eluted with Tris-HCl buffer pH 8.1 and retained material was desorbed with 0 2 M 1 4-diaminobutan in the same buffer

study of preformed EA\ -antibody complexes.

The results in the present study suggested the presence in RA-sera of both large and intermediate IC with C1q-fixing capacity These circulating IC can be isolated by the use of insolubilized C1q either because a substantial part of them are not saturated with C1 or they are bound to the C1q-resin as a result of replacement reaction solid-phase C1q exhibiting higher affinity for IC than in vivo bound C1 (25) Both IgM and IgG-RF's have been reported to fix C in their reaction with IgG (5 32) However serum IgG complexes were not precipitable with isolated C1q in contrast to the larger complexes in synovial fluids (28) Circulating IC exist in a high percentage of sero-positive RA-sera. IgG-complexes, up to 20 S in size, precipitable with monoclonal IgM RF's, were detected in 57 per cent of 42 RA-sera examined (29) Using the platelet aggregation technique (16) IgG complexes larger than 19S were detected in 24 per cent of sero-positive RA-sera and gel filtration data indicated the presence of intermediate IgG-complexes in 55–80 per cent of sero-positive sera (17)

The possibility that aggregated IgG contributed substantially to the C1q-bound material in the present study is less likely since the sera were kept at −20 C prior to testing. In addition, control runs were performed with similarly stored NHS

A variety of procedures for preparing insolubilized rabbit or human IgG (2, 4 5 13 17 18, 20 26) has been used in attempts to isolate antiglobulins from human sera. One recent approach has been to couple heat aggregated human IgG to cyanogen bromide-activated Sepharose (5 12) As observed in the present study (Table 2, Fig. 4) binding of RF's occurred also to Sepharose-coupled non-aggregated human IgG. Desorption of the antiglobulins has been achieved by use of acid buffers in previous studies. With regard to the elution of RF's (only ≤20 per cent of the RF activity of the original serum sample was recovered) from IgG-Sepharose resin in the present study it can not be concluded that the mild basic conditions on the columns (final pH in buffer 11 pH in eluted fractions around 9) contributed to the elution. The low affinity reported for anti-globulins (1 18) may also have facilitated the desorption.

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REFERENCES

1 *Abraham G N., Clark R. A & Vaughan J H.*: Characterization of an IgG rheumatoid factor binding properties and reactivity with the subclasses of human γG globulin. Immunochemistry 9 501 515 1972.

2. *Abrass J L. & Hemmer R.*: The measurement of IgG anti-IgG antibody in rheumatoid arthritis. J Immunol. Methods 4: 149–160 1974

3 *Agnello V., Koffler D., Eisenberg, J W., Winchester R. J & Kunkel H G.*: C1q precipitins in the sera of patients with systemic lupus erythematosus and other hypocomplementemic states characterization of high and low molecular weight types. J exp. Med. 134 228–241 1971

4 *Bianco N E., Panush R. S Stillman J S & S hur P H* Immunologic studies in juvenile rheumatoid arthritis. Arthr and Rheum. 14 685–696, 1971

5. *Bianco N E., Dobkin L. W & Sch P H* Immunological properties of isolated IgG and IgM anti-gamma-globulins (rheumatoid factors) Clin. exp. Immunol. 17 91 101 1974

6. *Bing D H* Purification of the first component of human complement by affinity chromatography on human γ-globulin linked to Sepharose. J Immunol. 107 1243–1249 1971

7 *Cuatrecasas P Wilchek M & Anfinsen C B* Selective enzyme purification by affinity chromatography Proc. N t. Acad. Sci. 61 636–643 1968

8. *Creighton W D Lambert P H & Miescher P A* Detection of antibodies and soluble antigen-antibody complexes by precipitation with polyethylene glycol. J Immunol. 111 1219–1227 1973

9 *Dixon F J* Pathogenesis of immunologic disease J Immunol. 109 187 192, 1972.

10 *Ewald R. W & S h bert A F.*: Agglutinating activity of the complement component C1q in the F II latex fixation test. J Immunol. 97 100 105 1966.

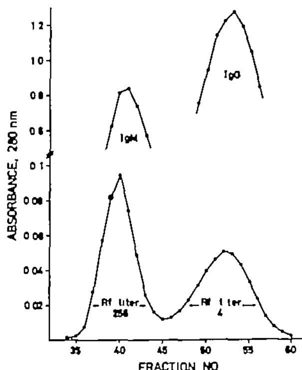


Fig 4 Gel filtration on a Sephadex G-200 column (1 4 × 180 cm) of material (●) desorbed from an IgG-Sepharose 6 B resin reacted with a RA serum Six fractions from the IgM peak and 9 fractions from the IgG peak were pooled separately concentrated to the same protein concentration and their latex agglutination titre determined. A sample of the encircled fraction was examined by electron microscopy Reference run with normal human serum (O) on the same G-200 column.

attempted by the procedure used for IC Immunodiffusion revealed only IgM and IgG in the desorbed material and its elution profile on a G-200 column, equilibrated with Tris-HCl buffer containing 0.2 M 1 4-diaminobutan is seen in Fig 4 Two pools were prepared of 6 fractions in the IgM region and 9 fractions contributing to the IgG peak The LA titers of the pools, concentrated to the same protein concentrations were 256 (IgM pool) and 4 (IgG pool) respectively The IgM in the IgM pool could be removed quantitatively by absorption with latex globulin particles. One fraction in the IgM peak, when examined in the electron microscope, revealed an abundance of IgM molecules and few contaminating molecules.

DISCUSSION

A variety of physical separation methods gradient centrifugation chromatography precipitation with polyethylene glycol or $(NH_4)_2SO_4$ and cryoprecipitation have been used in attempts to isolate circulating IC for further characterization. Biological methods of importance have been precipitation with monoclonal RF or C1q protein (3 29) It is particularly RA sera which have been analysed by use of these methods The primary purpose with the present study was not to characterize IC in RA-sera but rather to explore the potential use of resin bound C1q for IC isolation in general.

The method reported here for isolation of IC is similar to the procedure used by *Borg* (6) for purification of C1q although particularly in the work with RA-sera, we preferred to couple the C1q protein directly to the resin without an intermediate IgG linkage Binding of C1q to the activated Sepharose involves free amino groups on the protein. However critical C1q binding sites did not appear to be substantially affected by the conjugation process. When the use of insolubilized IgG in the immunosorbent step does not complicate subsequent analyses of desorbed material it may however be preferable to perform the IC isolation on a C1q-IgG resin. The disadvantage of this latter procedure is that both C1q and IC are desorbed by the diaminobutan-containing Tris buffer requiring a subsequent separation step. In addition assuming a C1q IgG molar ratio of 0 5 1 at saturating conditions we have estimated that only 10 to 20 per cent of the insolubilized IgG was available for binding of C1q

The use of a diaminoalkyl compound for desorption of IC was based on the earlier observation (6 30) that 1 4-diaminobutan interacts directly with C1q and inhibits the C1q-Ig reaction apparently by the titration of anionic groups on the C1q molecule (23) The usefulness of the outlined procedure for isolation of C-fixing complexes of viral antigen and antibodies was illustrated in the

Acta path. microbiol. scand. Sect. C, 84 53–58, 1976

EVALUATION OF THREE REVERSE PASSIVE HAEMAGGLUTINATION METHODS AND TWO RADIOIMMUNOASSAY TESTS TO BE USED FOR THE DETECTION OF HEPATITIS B SURFACE ANTIGEN

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Hansson, B. G. Evaluation of three reverse passive haemagglutination methods and two radioimmunoassay tests to be used for the detection of hepatitis B surface antigen. Acta path. microbiol. scand. Sect. C, 84 53–58, 1976.

Sensitivity and specificity of three reverse passive haemagglutination (RPHA) methods (Hepanosticon, Hepatest and Auscell) and of two solid-phase radioimmunoassays (RIA) (Ausria 125 and Ausria II 125) all phase 3 tests for hepatitis B surface antigen (HB_sAg) were compared with the sensitivity and specificity of an immunoelectroosmophoresis (IEOP) technique. By titration experiments the RPHA methods were shown to be 5–20 times more sensitive than the IEOP test, while RIA detected *5–10* times lower concentrations of antigen than the most sensitive RPHA test. In a study of sera drawn consecutively from patients with hepatitis B infections, the increased sensitivity of the test methods was according to the following order: IEOP, Hepanosticon, Hepatest, Ausria-125, Auscell, Ausria II 125. There were significant differences between all the methods except for that between Ausria-125 and Auscell. IEOP did not detect any false positives. If, however, RPHA tests were used, the incidence of nonspecific positive reactions would be in the range 0.5 per cent–0.9 per cent. The advantages of the individual test methods are discussed.

Key-words: Hepatitis B surface antigen; immunoelectroosmophoresis; reverse passive haemagglutination; radioimmunoassay.

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Since the time when the Australia antigen (now called hepatitis B surface antigen, HB_sAg) was discovered by *Blumberg et al.* (2) who used the Ouchterlony agar-gel immunodiffusion (ID), the methods later developed and used for the detection of the antigen have become increasingly sensitive. Immunoelectroosmophoresis (IEOP) (13) which is reported to be 10–30 times more sensitive than ID (1, 3, 6, 13) is still widely used as a screening test for HB_sAg. Certain variants of the very sensitive radioimmunoassay (RIA) technique to be used for the

11 *Farrell C Sogaard H & Svehag S-E* Detection of IgG aggregates or immune complexes using solid phase-C1q and protein A rich *Staphylococcus aureus* as an indicator system Scand J Immunol. *4* 673–680 1975

12 *Gaarder P I & Michaelsen T E.* Specificity of rheumatoid factors cross-reacting with human and rabbit IgG Acta path. microbiol scand Sect. B *22* 733–741 1974

13 *Hier O D & Turner M W* Determination of IgG anti-globulin in rheumatoid disease using insolubilized human IgG Clin exp Immunol *15* 93–101 1973

14 *Jewell D P & MacLennan I C M* Circulating immune complexes in inflammatory bowel disease Clin. exp. Immunol. *14* 219–226 1973

15 *Leber P D & McCluskey R T* Immune complex diseases. In Zweifach, B. W., Grant, L. & McClusky R. T (Eds.) The inflammatory Process. Academic Press, New York *III* 1974 pp 401–441

16 *Myllylä G Vaheri A & Penttinen K* Detection and characterization of immune complexes by the platelet aggregation test. Clin exp Immunol *8* 399–408, 1971

17 *Norberg R* IgG complexes in serum of rheumatoid arthritis patients. Scand. J Immunol *3* 229–236 1974

18 *Normansell D E.* Anti-γ-globulins in rheumatoid arthritis sera. *II The reactivity of* anti-γ-globulin rheumatoid factors with altered γG-globulin Immunochemistry *8* 593–602 1971

19 *Onyewotu I I Holborow E J & Johnson G D* Detection and radioassay of soluble circulating immune complexes using guinea pig peritoneal exudate cells. Nature *248* 156–159 1974

20 *Panush R S Bianco N E & Schur P H* Serum and synovial fluid IgG IgA and IgM antigammaglobulins in rheumatoid arthritis. Arthr and Rheum. *14* 737–747 1971

21 *Radwan A I & Burger D* The complement requiring neutralization of equine arteritis virus by late antisera. Virology *51* 71 77 1973

22 *Ropes M I Bennett G A, Cobb S Jacox R & Jessar R A* 1958 revision of the diagnostic criteria for rheumatoid arthritis. Arthritis Rheum. *2* 16–18, 1958.

23 *Sledge C R & Bing D H.* Binding properties of the human complement protein C1q. J. Biol. Chem. *248* 2818–2823 1973

24 *Svehag S E Manhem L & Bloth B.* Ultrastructure of human C1q protein Nature New Biol *238* 117–118, 1972.

25 *Svehag S E* Unpublished data.

26 *Torrigiani G & Roitt I M.* Antiglobulin factors in sera from patients with rheumatoid arthritis and normal subjects. Quantitative estimation in different immunoglobulin classes. Ann Rheum. Dis. *26* 334–340 1967

27 *Theofilopoulos A N Wilson C B. Bokisch V A & Dixon F J* Binding of soluble immune complexes to human lymphoblastoid cells. J exp Med. *140* 1230–1244 1974

28 *Winchester R Agnello V & Kunkel, H G.* The joint fluid γG-globulin complexes and their relationship to intraarticular complement diminution. Ann. N Y Acad. Sci *168* 195–203 1969

29 *Winchester R J Kunkel H G & Agnello V.* Occurrence of γ-globulin complexes in serum and joint fluid of rheumatoid arthritis patients use of monoclonal rheumatoid factors as reagents for their demonstration. J exp. Med. *134* 286–295 1971

30 *Wirtz G H* Influence of alliphatic amines on the interaction of human C'_1 with eac 4 Immunochemistry *2* 95–102 1965

31 *Yonemasu K & Stroud R M* C1q Rapid purification method for preparation of monospecific antisera and for biochemical studies. J Immunol *106* 304–313 1971

32 *Zvaifler N J & Schur P H* Reactions of aggregated mercaptoethanol treated gamma globulin with rheumatoid factor-precipitin and complement fixation studies. Arthr and Rheum. *11* 523–536 1968.

TABLE 1 *Relative Sensitivity of Six Different Test Methods for Detecting HB_sAg. The Results Were Obtained by Testing Six Two-step Dilution Series Each of HB_sAg/ad and HB_sAg/ay*

Test method	Relative sensitivity
IEOP	1.0
Hepanosticon	4.5
Hepatest	6.8
Ausell	18
Austria–125	100
Austria II–125	240

in a water bath at 45 C. The rinsing procedure described above is repeated. The beads are transferred to counting tubes and measured in a gamma counter. The entire test can be carried out within five hours.

RESULTS

Sensitivity of tests in dilution experiments. The RPHA tests showed antigen titres between those of IEOP and RIA, Austria II–125 being more sensitive than Austria–125. No difference in sensitivity regarding HB_sAg/ad and HB_sAg/ay was found with any of the six test methods (Table 1).

None of the ten strongly positive sera from dialysis patients showed negative prozone when tested by the three RPHA methods.

Local HB_sAg reference panel. Using IEOP 36 of the positive samples in this reference panel were detected. All of the 42 positive sera were detected if Ausell or Austria–125 were used, while Hepatest failed to detect one and Hepanosticon two of the 42 positive sera. This reference panel was not tested with Austria II–125.

Blood samples consecutively drawn from patients with acute hepatitis B. In the 20 patients with serial bleedings, the length of the time after onset of jaundice, during which HB_sAg could be detected, varied, depending on the test used. The results are illustrated in Fig. 1. Using IEOP 50 per cent of the patients were still found to be positive for HB_sAg 26 days after onset of jaundice. If Hepanosticon and Hepatest were used, HB_sAg could be detected throughout a longer period of time, covering in total about 38 days, while HB_sAg still could be detected in 50 per cent of the patients about 48 days after onset of jaundice if Ausell or the two RIA methods were used. Samples from all of the 20 patients turned negative for HB_sAg irrespective of the test method used. When the number of positive samples was compared by Wilcoxon's signed-rank test (21) Hepanosticon was found to detect significantly more positives than IEOP ($P < 0.001$), Hepatest significantly more positives than Hepanosticon ($P < 0.001$) and Ausell significantly more positives than Hepatest ($P < 0.001$). Austria II–125 was more sensitive than Ausell, but the difference had a lower degree of significance ($P = 0.05$). No difference in sensitivity of Ausell and Austria–125 was seen ($P > 0.2$) while Austria II–125 detected significantly more positive samples than Austria–125 ($P = 0.005$).

Cases of acute hepatitis. Among the 102 cases of acute hepatitis which by the IEOP test were found to be negative for HB_sAg Austria–125 revealed that 17 were positive. Ausell and Hepatest found 16 and 4 positive cases, respectively, while Hepanosticon failed to reveal any.

Specificity analysis. Within the recent five-year-period, more than 65,000 samples obtained from blood donors have been tested for HB_sAg by IEOP in this laboratory. So far no false positive results have been obtained (8). Sera from 450 first time donors were tested by the RPHA tests (Table 2). Hepanosticon showed four, Hepatest three and Ausell three agglutinating sera. None of these sera were found to be positive by more than one method. The confirmatory tests of Hepanosticon and Hepatest showed that the samples found positive by these methods were negative for HB_sAg. Two out of the three sera found to be positive by Ausell were negative when tested by Austria II 125 while the third serum was positive also when tested by Austria II–125 and showed identity with HB_sAg in a neutralization test. As reported in a previous study (8) 0.5 per cent false

detection of HB_sAg have also been developed and by now they are used in many laboratories (10 12 20) Recently three different reverse passive haemagglutination (RPHA) tests for HB_sAg have been introduced i e. Hepanosticon* (17), Hepatest** (3) and Auscell*** (4) In these tests, erythrocytes have been sensitized with anti HB antibodies. When mixed with material containing HB_sAg the cells are agglutinated

It has been suggested that phase 1 (ID) phase 2 (IEOP complement fixation and other methods of similar sensitivity) and phase" 3 (RPHA and RIA) should be utilized in descriptions of the three categories of procedures, in order of increasing sensitivity instead of first second and third generation" because of the inherent biological implications associated with the latter term (18) The aim of this work is to investigate the sensitivity and specificity of three commmercial RPHA methods which until now to our knowledge have not been done in the same study and to compare with two commercial RIA tests and the IEOP used in the routine in this laboratory

MATERIALS AND METHODS

Test materials a) Six samples of HB Ag/ad and six samples of HB Ag/ay were titrated and tested by three RPHA and two RIA methods. b) Ten sera from dialysis patients with high concentration of HB Ag were tested by the three RPHA methods. c) A local reference panel consisting of sera containing different amounts of HB_sAg namely 13 of subtype ad 29 of subtype ay and 10 negative controls, was investigated by the different methods d) Serum samples, consecutively drawn at intervals of about one week from 20 patients with acute hepatitis B were tested for HB_sAg e) Sera from 102 cases clinically diagnosed as acute hepatitis even though IEOP test for HB Ag was negative, were also tested by the three RPHA methods and one RIA method The serum samples were collected during the first two weeks after onset of jaundice

Blood donor sera In order to assess the incidence of false positive results, 430 sera from first-time blood donors were tested by the RPHA methods.

Immunoelectroosmophoresis (IEOP) These tests were performed as described in a previous work (6) The technique was controlled by the "Reference hepatitis B antigen panel no 2" from the Bureau of Biologics, NIH Bethesda, Md, as previously reported (7)

Hepanosticon The test has been described by *Reesink et al.* (16) and by *Schuurs & Kacaki* (17) Formalin stabilized sheep erythrocytes coated with anti-HB antibodies from sheep are supplied in lyophilized form. Reconstituted cells can be stored frozen at − 20 C and thawed as needed. The test is ready after three hours. Most of the unspecifically agglutinating factors can be eliminated by absorption with sheep erythrocytes coated with sheep gammaglobulin devoid of anti-HB

Hepatest In this test, which has been developed by *Cayzer et al.* (3) turkey erythrocytes coated with equine anti HB are used. The cells are supplied in lyophilized form. Reconstituted samples can be frozen at − 20 C and thawed later when needed. The sedimentation pattern can be read after ½–1 hour In order to reveal non-specific anti-turkey cell reactivity the agglutinating sera are controlled by erythrocytes coated with normal horse globulin

Auscell The test is supplied in the form of lyophilized human erythrocytes sensitized with anti-HB from guinea pigs (4) If kept at + 4 C, the cells can be used for up to 14 days after reconstitution The test is read after two hours. All specimens found to be positive by the screening test can be controlled by a confirmatory neutralization test. In the present investigation, Ausria II 125 was used as the confirmatory test.

Ausria -125 was the first solid-phase RIA to be introduced by Abbott Laboratories. The solid phase consists of polypropylene tubes coated with guinea pig anti HB Performance of the test covers in total five hours at least. The test has been described by *Ling & Overby* (12)

Ausria II 125 represent an improvement of Ausria–125 Polypropylene beads coated with guinea pig anti-HB replace the tubes. The beads are dispensed into the wells of a reaction tray provided in the test kit. Patient serum (0 2 ml) is added into the wells, and the tray is incubated in a water bath at 45 C for two hours. Using a semi-automated aspiration and rinsing system, a total of 10 ml of distilled water is sufficient for the washing of each well and bead After the rinsing procedure 0 2 ml human ^{125}I anti HB is added to each well and the tray is incubated for one hour

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Reference HB Ag/ad and HB Ag/ay were kindly supplied by Dr *J E Nutter* NIH, Bethesda, Md, U.S.A.

compared to IEOP has also been demonstrated by *Wallace et al* (19). However no differentiation between the individual RPHA methods was done, nor was the sensitivity of the IEOP technique used discussed.

The relatively high incidence of nonspecific positive results obtained by RPHA calls for specificity analysis of all samples primarily held to be positive. In order to establish identity with HB_sAg, confirmatory inhibition by anti-HB should be performed. The confirmatory tests of Hepanosticon and Hepatest reduce most of the unspecific results, but fail to show evidence of a specificity which is the case as regards the control test of Auscell. Neutralization test had to be performed in the case of the previously used Austria 125 and seems to be necessary also if Austria II-125 is used, by which two sera that could not be neutralized by anti-HB, have been found (9).

Among the methods studied in the present work, the RPHA tests are most simple and rapid. The RIA technique has the advantage of objective reading of the results compared with the subjective reading of the RPHA tests. In the case of Hepanosticon and Hepatest, reconstituted test cells can be frozen and saved to be used later, and the reconstituted Auscell test cells can be kept for two weeks at +4 C, in other words, waste of test material is not necessary. The performance of Austria II-125 is much easier than the performance of Austria-125 but it is more laborious and time-consuming than the RPHA tests. Besides, expensive equipment, a certain amount of wasted test material and the high number of controls increase the cost per RIA test. As all reagents to be used in the IEOP test are produced and controlled in this laboratory, the cost of the material to be used per test is very low (6). In a previous work (8) it was demonstrated that the rate at which HB_sAg was detected among first-time blood donors increased from the 0.5 per cent detected by IEOP to 0.6 per cent if Austria-125 was used. Furthermore, if one case of transfusion hepatitis was to be prevented by screening of all samples obtained from blood donors by Austria-125, the costs were estimated to amount to at least 140,000 Swedish kronor (the equivalent of U.S. $ 30,000). Accordingly, the additional sensitivity of Austria-125 was considered to be too expensive to be of practical value in routine examinations of all samples from a blood donor population like the one studied. Thus, apart from sensitivity, factors such as costs, handiness and type of blood donor population may influence the choice of test method. It is beyond discussion, however, that all of the three RPHA tests are superior to the commercial IEOP kits and should replace those, unless application of Austria II-125 is indicated.

The skilful technical assistance of Mrs *Zita Berlin* is gratefully acknowledged.

REFERENCES

1. *Alter, H. J., Holland, P. V. & Purcell, R. H.:* Counterelectrophoresis for detection of hepatitis-associated antigen: methodology and comparison with gel diffusion and complement fixation. J. Lab. Clin. Med. *77*: 1000-1010, 1971.
2. *Blumberg, B. S., Alter, H. J. & Visnich, S.:* A "new" antigen in leukemia sera. J. Amer. med. Ass. *191*: 541-546, 1965.
3. *Cayzer, I., Dane, D. S., Cameron, C. H. & Denning, J. V.:* A rapid haemagglutination test for hepatitis-B antigen. Lancet *i*: 947-949, 1974.
4. *German, K. H., Sturdivant, S. A. & Rightsel, W. A.:* Evaluation of a red cell aggl tination test for detection of Australia antigen (HBAg). Appl. Microbiol. *25*: 524-527, 1973.
5. *Gocke, D. J. & Howe, C.:* Rapid detection of Australia antigen by counterimmunoelectrophoresis. J. Immunol. *104*: 1031-1032, 1970.
6. *Hansson, B. G. & Johansson, T.:* Improved technique for detecting Australia antigen by immunoelectroosmophoresis. Vox Sang. *21*: 531-539, 1971.
7. *Hansson, B. G. & Johansson, T.:* Evaluation of solid phase radioimmunoassay (Austria-125) for the detection of hepatitis B antigen (Australia antigen) in two clinical materials. J. clin. Path. *27*: 35-39, 1974.
8. *Hansson, B. G., Sundström, G. & Johansson, T.:* Occurrence of hepatitis B surface antigen (HB_sAg) in blood donors and patients suffering from transfusion hepatitis. Acta path. microbiol. scand. Sect. C *83*: 112-120, 1975.

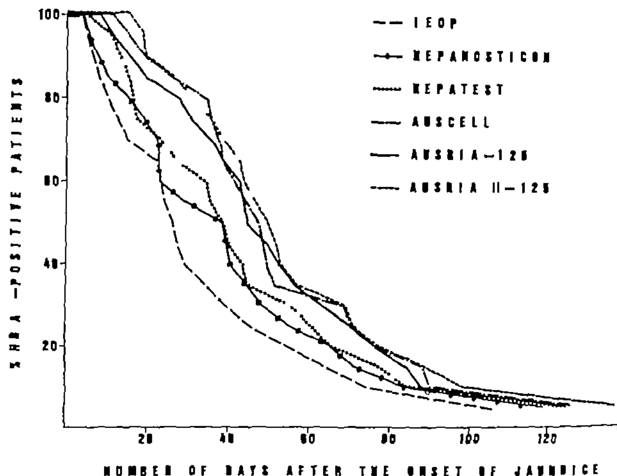


Fig 1 Comparison of six test methods to be used for the detection of HB_sAg. Blood samples serially drawn from 20 patients with acute hepatitis B were tested.

positives were found by Ausria 125 in samples obtained from first time blood donors this result is included in Table 2

DISCUSSION

The sensitivity of the RPHA tests were successively increasing according to the following order Hepanosticon Hepatest and Auscell while Ausria II-125 was the most sensitive test. In the case of clinical materials Auscell and Ausria-125 showed about the same sensitivity In the titration experiments only minor differences between the three RPHA methods were seen. The RIA tests however detected six to 13 times lower concentrations of antigen than Auscell (Table 1) These results are in accord with those obtained by *Lange et al* (11) but disagree with a report by *Peterson et al* (14) which exhibited roughly the same sensitivity for Auscell and Ausria-125 in dilution experiments. In tests of the consecutive serum samples, Auscell which was shown to be the most sensitive RPHA test was as sensitive as Ausria-125 which is in fair accord with the findings by *Peterson et al* (14) and *Lange et al* (11) The higher sensitivity of RPHA

TABLE 2 Incidence of False Positive Samples Obtained by Five Different Test Methods Used for the Detection of HB_sAg

Test method	No of false positives/no. of blood donor sera tested
IEOP	0/> 65 000
Hepanosticon	4/430 (0 9 %)
Hepatest	3/430 (0 7 %)
Auscell	2/430 (0.5 %)
Ausria-125	9/1 742 (0.5 %)

compared to IEOP has also been demonstrated by *Wallace et al.* (19). However no differentiation between the individual RPHA methods was done nor was the sensitivity of the IEOP technique used discussed.

The relatively high incidence of nonspecific positive results obtained by RPHA calls for specificity analysis of all samples primarily held to be positive. In order to establish identity with HB_sAg, confirmatory inhibition by anti-HB should be performed. The confirmatory tests of Hepanosticon and Hepatest reduce most of the unspecific results, but fail to show evidence of a specificity which is the case as regards the control test of Auscell. Neutralization test had to be performed in the case of the previously used Austria 125 and seems to be necessary also if Austria II 125 is used, by which two sera that could not be neutralized by anti HB_s have been found (9).

Among the methods studied in the present work, the RPHA tests are most simple and rapid. The RIA technique has the advantage of objective reading of the results compared with the subjective reading of the RPHA tests. In the case of Hepanosticon and Hepatest, reconstituted test cells can be frozen and saved to be used later and the reconstituted Auscell test cells can be kept for two weeks at +4 C in other words, waste of test material is not necessary. The performance of Austria II 125 is much easier than the performance of Austria–125 but it is more laborious and time-consuming than the RPHA tests. Besides, expensive equipment, a certain amount of wasted test material and the high number of controls increase the cost per RIA test. As all reagents to be used in the IEOP test are produced and controlled in this laboratory, the cost of the material to be used per test is very low (6). In a previous work (8), it was demonstrated that the rate at which HB_sAg was detected among first-time blood donors increased from the 0.5 per cent detected by IEOP to 0.6 per cent if Austria–125 was used. Furthermore if one case of transfusion hepatitis was to be prevented by screening of all samples obtained from blood donors by Austria–125 the costs were estimated to amount to at least 140,000 Swedish kronor (the equivalent of U.S. $ 30,000). Accordingly the additional sensitivity of Austria–125 was considered to be too expensive to be of practical value in routine examinations of all samples from a blood donor population like the one studied. Thus, apart from sensitivity factors such as costs, handiness and type of blood donor population may influence the choice of test method. It is beyond discussion however that all of the three RPHA tests are superior to the commercial IEOP kits and should replace those, unless application of Austria II–125 is indicated.

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REFERENCES

1. *Alter H J., Holland P V & Purcell, R. H.* Counterelectrophoresis for detection of hepatitis-associated antigen methodology and comparison with gel diffusion and complement fixation. J. Lab. Clin. Med. 77 1000–1010 1971
2. *Blumberg, B S Alter H J & Visnich S.* A "new" antigen in leukemia sera. J Amer med Ass. *191* 541 546, 1965.
3. *Cayzer I Dane D S Cameron C H & Denning, J V* A rapid haemagglutination test for hepatitis-B antigen. Lancet 947–949 1974
4. *Germeau K H., Sturdivant S K & Rightsel W A* Evaluation of a red cell agglutination test for detection of Australia antigen (HBAg) Appl Microbiol. 25 524 527 1973
5. *Gocke D J & Howe C* Rapid detection of Australia antigen by counterimmunoelectrophoresis. J Immunol. *104* 1031 1032, 1970.
6. *Hansson B. G & Johnsson T* Improved technique for detecting Australia antigen by immunoelectroosmophoresis. Vox Sang. *21* 531–539 1971
7. *Hansson B G & Johnsson T* Evaluation of a solid phase radioimmunoassay (Austria–125) for the detection of hepatitis B antigen (Australia antigen) in two clinical materials. J clin Path *27* 35–39 1974
8. *Hansson B. G Sundström G & Johnsson T* Occurrence of hepatitis B surface antigen (HB_sAg) in blood donors and patients suffering from transfusion hepatitis. Acta path. microbiol. scand Sect. C, *83* 112–120 1975

Hansson B G & Johansson T Unpublished data.

Hollinger F B Vorndam V & Dreesman G R Assay of Australia antigen and antibody employing double-antibody and solid phase radioimmunoassay techniques and comparison with the passive hemagglutination methods. J Immunol *107* 1099–1111 1971

Lange W Köhler H & Apodaca J Der Nachweis von Hepatitis-B-(Surface-) Antigen. Dtsch. med Wsch. *100* 508–512, 1975

Ling C M & Overby L R Prevalence of hepatitis B virus antigen as revealed by direct radioimmune assay with ^{125}I-antibody J Immunol *109* 834–841 1972

Pesendorfer F Krassnitzky O & Wewalka F Immunoelektrophoretischer Nachweis von "Hepatitis associated antigen" (Au/SH-antigen) Klin Wschr *48* 58–59 1970

Peterson D A Froesner G G & Deinhardt F W Evaluation of passive hemagglutination, solid-phase radioimmunoassay and immunoelectroosmophoresis for the detection of hepatitis B antigen. Appl. Microbiol *26* 376–380 1973

Prince A M & Burke K Serum hepatitis antigen (SH) Rapid detection by high voltage immunoelectroosmophoresis. Science *169* 593–595 1970

16 *Reesink H W Duimel W J & Brummelhuis H G J* Evaluation of a new haemagglutination technique for the demonstration of hepatitis-B antigen Lancet *ii*. 1351–1353, 1973

17 *Schuurs A H W M & Kacaki J* Reversed haemagglutination test for the detection of hepatitis B antigen (HBAg) Vox sang. *25* 97–104 1974

18 Symposium on viral hepatitis. International association of biological standardization, Milan 1974

19 *Wallace J Barr A & Milne G R* Which techniques should be used to screen blood donations for Hepatitis B surface antigen? Brit. med J *2* 412–414 1975

20 *Walsh J H Yalow R & Berson S A* Detection of Australia antigen and antibody by means of radioimmunoassay techniques. J Infect. Dis. *121* 550–554 1970

21 *Wilcoxon F* Individual comparisons by ranking methods. Biometrics *1* 80–83 1945

Acta path microbiol scand Sect. C, 84 59-67 1976

SURFACE STRUCTURES INFLUENCING THE MIGRATION *IN VIVO* (ECOTAXIS) OF MURINE THYMOCYTES, T AND B-CELLS

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Wiig, J N Surface structures influencing the migration *in vivo* (ecotaxis) of murine thymocytes, T and B-cells. Acta path microbiol. scand. Sect. C, 84 59-67 1976.

The effect of protease and neuraminidase treatment on the ecotaxis of radioactively labelled thymocytes, T and B-cells was studied by scintillation counting and autoradiography Each enzyme had a similar effect on all cell types. Both protease and neuraminidase treatment strongly reduced the migration to the lymph nodes while the distribution to the spleen was more profoundly reduced by neuraminidase than by protease In contrast, a similar and less pronounced effect of the enzymatic treatments on the circulation to the Peyers patches was found. The possible structure of the surface "receptors" involved is discussed in view of the known effects of these enzymes on the cell membranes

Key words Murine thymocytes T-cells B-cells migration.

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In addition to functional and structural differences, thymocytes, T and B-cells show *specific* migration patterns to the different lymphoid organs. This capacity has been named ecotaxis by *de Sousa* (11) A minority of thymocytes tends to go to the paracortical areas of lymph nodes while the majority go to the periarteriolar areas of the white pulp of spleen (6) T-cells migrate to the same areas though in more equal proportions (9, 23) and B-cells to the outer cortex and medulla of lymph nodes and the outer part of the white pulp of spleen (8) In rats, the ecotaxis of thymocytes, thoracic duct and lymph node cells has been shown to be changed by treatment of the cells with trypsin and neuraminidase (2, 20, 21) In chickens, a similar effect has been found after neuraminidase treatment of thymocytes and blood lymphocytes (3) and in mice, it has been shown that infecting animals with the neuraminidase containing Newcastle disease virus causes depletion of the thymus dependent areas of lymphoid organs as well as lymphopenia (22) From these studies it has been concluded that ecotaxis depends on some kind of "receptor" on the lymphoid cell membrane. It has been suggested that the change in ecotaxis caused by both trypsin and neuraminidase treatment is due to the removal of sialic acid molecules from such "receptors" (20)

The present study was undertaken to test the effect of protease and neuraminidase treatment on the ecotaxis of murine thymo-

9 *Hansson B G & Johansson T* Unpublished data.
10 *Hollinger F B Lorndam V & Dreesman G R* Assay of Australia antigen and antibody employing double antibody and solid phase radioimmunoassay techniques and comparison with the passive hemagglutination methods J Immunol *107* 1099–1111 1971
11 *Lange W Köhler H & Apodaca J* Der Nachweis von Hepatitis-B-(Surface-) Antigen Dtsch. med Wsch. *100* 508–512 1975
12 *Ling C M & Overby L R* Prevalence of hepatitis B virus antigen as revealed by direct radioimmune assay with [125]I antibody J Immunol *109* 834–841 1972
13 *Pesendorfer F Krassnitzky O & Wewalka F* Immunoelektrophoretischer Nachweis von "Hepatitis associated antigen" (Au/SH antigen) Klin. Wschr *48* 58–59 1970
14 *Peterson D A Froesner G G & Deinhardt F W* Evaluation of passive hemagglutination, solid-phase radioimmunoassay and immunoelectroosmophoresis for the detection of hepatitis B antigen. Appl Microbiol. *26* 376–380 1973
15 *Prince A M & Burke K* Serum hepatitis antigen (SH) Rapid detection by high voltage immunoelectroosmophoresis. Science *169* 593–595 1970.
16 *Reesink H W Duimel W J & Brummelhuis H G J* Evaluation of a new haemagglutination technique for the demonstration of hepatitis-B antigen. Lancet *ii* 1351 1353, 1973
17 *Schuurs A H W M & Kacaki J* Reversed haemagglutination test for the detection of hepatitis B antigen (HBAg) Vox sang. *25* 97–104 1974
18. Symposium on viral hepatitis, International association of biological standardization, Milan 1974
19 *Wallace J., Barr A & Milne G R* Which techniques should be used to screen blood donations for Hepatitis B surface antigen? Brit. med J *2* 412–414 1975
20 *Walsh J H Yalow R. & Berson S A.* Detection of Australia antigen and antibody by means of radioimmunoassay techniques. J Infect. Dis. *121* 550–554 1970
21 *Wilcoxon F* Individual comparisons by ranking methods. Biometrics *1* 80–83, 1945

TABLE 1 *Distribution Pattern of C-labelled Thymocyt Treat d with Prot ase Neu minidase at 4 and 24 Hours after I jection. Recover d Am t are Gi en as Percent g of Radioactivity I jected ± Standard Deviatio*

Organs	Control		Heat killed		Protease treated		Neuraminidase treated	
	4h	24h	4h	24h	4h	24h	4h	24h
Lymph nodes	0.7 ± 0.1	0.6 ± $	0.1 ± $	0.1 ± $	0.1 ± $	0.1 ± $	0.1 ± $	0.1 ± $
Spleen	18.5 ± 3.1	19.1 ± 2.4	1.4 ± 0.2	1.5 ± 0.3	11.6 ± 2.2	10.0 ± 1.8	3.3 ± 0.4	2.0 ± 0.5
Thymus	‡		—			—	—	—
Small intestine	0.6 ± 0.1	0.2 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.2 ± $	0.3 ± 0.1	0.2 ± 0.1
Lungs	3.0 ± 0.8	0.3 ± $	3.0 ± 0.9	1.0 ± 0.1	1.6 ± 0.4	0.2 ± $	1.9 ± 0.3	0.8 ± 0.1
Liver	19.7 ± 2.8	~0.2 ± 2.3	17.2 ± 1.9	17.5 ± 2.1	34.0 ± 4.9	32.9 ± 4.0	21.8 ± 2.9	18.3 ± [illegible]
Total recovery	42.5 ± 1.8	40.4 ± 1.0	22.5 ± 1.9	20.6 ± 1.7	48.0 ± 4.2	43.4 ± 2.3	27.4 ± 3.7	21.4 ± 1.5

Hours after jection of cell ‡ Minute amounts recovered. $ Less than 0.1

TABLE 2a. *Distribution of Cr-labelled T-cells from Spleens of Cyclophosphamide Injected Animals Treated with Protease N uraminidase at 4 and 24 Hour after I.v. I jection. R ered Amounts are Given as Per entage f Radioact vity I jected ± Standard D iation*

Organs	Control		Heat killed		Protease treated		Neuraminidase treated	
	4h	24h	4h	24h	4h	24h	4h	24h
Lymph nodes	5.1 ± 1.2	5.9 ± 1.2	0.3 ± $	0.4 ± 0.1	1.7 ± 0.2	3.3 ± 1.1	1.3 ± 0.3	1.0 ± 0.2
Spleen	15.2 ± 2.2	13.3 ± 1.7	0.7 ± 0.1	1.0 ± 0.5	12.8 ± 1.2	12.7 ± 2.0	8.5 ± 1.5	8.0 ± 1.1
Thymus	—‡	—	—	—	—	—	—	—
Small intestine	1.2 ± 0.3	1.0 ± 0.2	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
Lungs	6.0 ± 1.4	2.2 ± 0.6	7.5 ± 1.4	2.3 ± 0.4	5.3 ± 1.1	1.2 ± 0.3	2.7 ± 0.7	0.9 ± 0.1
Liver	18.4 ± 2.3	19.0 ± 2.4	19.9 ± 1.3	19.7 ± 3.0	18.5 ± 7.2	18.1 ± 6.7	34.0 ± 3.0	~7.9 ± 2.7
Total recovery	45.9 ± 2.3	43.4 ± 1..	28.7 ± 1.4	23.8 ± 2.9	38.8 ± 7.5	36.0 ± 6.1	47.4 ± 3.2	38.3 ± 1.7

Hours after I. Injection of cells. ‡ Minute amounts recovered. $ Less than 0.1

cytes, T and B-cells. The effects are discussed in the light of previously shown effects on surface structures of such cells.

MATERIALS AND METHODS

Mice C3H/A/BOM male mice 8–10 weeks old from Gamle Bomholtgaard, Laven Denmark, were used

Reagents Neuraminidase was obtained from Behringwerke AG Marburg am Lahn, Germany Protease (P 5130 GR VI) from Sigma Chemical Company St. Louis, USA. Sodium chromate of ^{51}Cr from the Institute of Atomic Energy Kjeller Norway and ^{3}H adenosine from the Radio Chemical Center Amersham England Rabbit anti mouse immunoglobulin serum was provided by Mr *S Helgeland* Broegelman Research Institute Bergen, Norway It was prepared from anti mouse full serum by means of an immunoglobulin immunoabsorbant column (5) Fluorescein isothiocyanate conjugated goat anti rabbit Immunoglobulin serum was prepared as described (13)

Cell suspensions Single cell suspensions were prepared as previously described (16) Haemolysis was obtained by washing with 0 83 per cent NH_4Cl and twice with Hanks balanced salt solution (HBSS) (19)

Thymocytes prepared from normal thymus belong to one major spleen seeking population and a *minor lymph node seeking one (6) T-cells* were obtained from either spleens of cyclophosphamide injected mice (T-spleens) which contain about 85 per cent theta positive cells (15 16) or by a nylon wool filtration technique of lymph node cells (brachial axillary inguinal and mesenterial) modified from (14) In the present study the percentage of Immunoglobulin positive B-cells was reduced from 19 per cent to 1 per cent by column passage (vide infra) *B-cells were prepared from the spleens of* adult thymectomized lethally irradiated and bone marrow reconstituted mice (B-spleens) which contain about 80 per cent B-cells (10)

Fluorescence testing An indirect test was performed with rabbit anti-mouse immunoglobulin antibodies and fluorescein isothiocyanate conjugated goat anti rabbit immunoglobulin serum Incubations were performed for 30 min at 4 C and the cells were washed *3 times after each* incubation in HBSS with added 0 03 M sodium azide to prevent capping and endocytosis. Mounting and microscopy was carried out as previously (19)

Radioactive labelling The cells were incubated at a concentration of 100 10^6 cells/ml with added 100 μ Ci ^{51}Cr for the scintillation counting or 10 μ Ci ^{3}H-adenosine/ml for the autoradiography studies for 60 min at 37 C under constant agitation *The cells were then* washed twice in HBSS with added 0 005 M $CaCl_2$.

Enzymatic treatment Subsequent to radioactive labelling the cells were subjected to enzymatic treatment. To 100 10^6 cells in 1 ml HBSS with 0 005 M $CaCl_2$ was added 0 125 mg protease plus 0 100 mg deoxyribonuclease or 100 units of neuraminidase The cells were incubated at 37 C for 30 min and 60 min *respectively as previously de*scribed (17 18) Control cells were incubated at 37 C for 30 min. Cells were killed by heating to 55 C for 30 min These cells were 75 per cent 95 per cent trypan blue positive.

Preparation of recipient animals Viability of the cells was tested prior to i.v. injection. Control and enzyme treated suspensions containing more than 20 per cent non viable cells, as estimated by the trypan blue exclusion technique, were discarded The cell suspensions were diluted with HBSS and 20 10^6 cells in 0 5 ml were injected, after prewarming of the animals, into the tail vein of groups of five These recipients were killed at 4 and 24 hours, 3 and 2 at a time and the following organs removed lymph nodes (brachial, axillary *inguinal mesenterial) spleen, thymus*, small intestine lungs and liver

Scintillation counting was performed in a Nuclear enterprise automatic β-γ-counter (NE 8312) on separate organs or pooled lymph nodes and *three 10 µl samples of the injected cell suspensions.* Three experiments were performed on the cells of both kinds of T-cells suspensions. Otherwise two experiments were performed.

Autoradiography The organs were fixed in cold buffered formalin embedded in paraffin and sectioned at 4μ. Autoradiographs were prepared using Kodak strip film. The sections were developed after an exposure time of 3 weeks and stained with Haematoxylin/Eosin. Autoradiographs of the injected cell suspensions were prepared on cytocentrifuge preparations in a similar manner except that the staining was performed with May-Grunwald Giemsa. *Cells with more than 10 grains* were considered positive. About 80 per cent of the cells in the suspensions gave this count.

RESULTS

Control cells Estimated by scintillation counting the main differences in ecotaxis pattern *between the cell types were shown* by differences in the lymph node uptake (Fig 1)

The injected thymocytes were found mainly in the spleen and liver while a small proportion was located *in the lymph nodes and* small intestine (Table 1) Only minute amounts of radioactivity were recovered from the thymus.

T-cells prepared from cyclophosphamide

TABLE 3. *Distribution Pattern of Cr-labelled B-cells from Spleens of Adult Thymectomized Lethally Irradiated and Bone Marrow Reconstituted Animals Treated with Protease or Neuraminidase at 4 and 24 Hours after I.v. Injection. Recovered Amounts are Given as Percentage of Radioactivity Injected ± Standard Deviation*

Organs	Control		Heat killed		Protease treated		Neuraminidase treated	
	4h	24h	4h	24h	4h	24h	4h	24h
Lymph nodes	0.3 ± $	0.5 ± $	0.1 ± $	0.1 ± $	0.1 ± $	0.1 ± $	0.1 ± $	0.1 ± $
Spleen	18.2 ± 2.7	14.4 ± 2.8	1.0 ± 0.1	1.1 ± 0.1	11.6 ± 1.9	12.8 ± 1.7	7.8 ± 1.2	6.5 ± 0.3
Thymus	§	–		–	–	–	–	–
Small intestine	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.3 ± $
Lungs	5.1 ± 0.1	0.7 ± 0.1	5.0 ± 1.0	1.3 ± 0.1	2.7 ± 0.9	0.3 ± $	4.3 ± 0.5	0.8 ± $
Liver	16.6 ± 2.8	15.1 ± 2.3	13.5 ± 2.1	21.8 ± 2.0	27.8 ± 3.7	29.9 ± 1.9	26.5 ± 3.0	26.0 ± 3.7
Total recovery	38.9 ± 1.8	31.1 ± 0.4	20.4 ± 1.1	24.7 ± 1.6	42.9 ± 4.3	43.3 ± 3.7	38.8 ± 2.1	33.7 ± 4.4

Hours after i.v. injection of cells. § Minute amounts recovered. $ Less than 0.1

TABLE 2b *Distribution of ⁵¹Cr-labelled T-cells from Lymph Nodes Fractionated on a Nylon Wool Column and Treated with Protease or Neuraminidase at 4 and 24 Hours after i.v. Injection Recovered Amounts are Given as Percentage of Radioactivity Injected ± Standard Deviation*

Organs	Control		Heat killed	Protease treated	Neuraminidase treated	
	4h*	24h	4h	4h	4h	24h
Lymph nodes	3.8 ± 1 0	3 9 ± 1 1	0 1 ± ‡	0 1 ± ‡	0 2 ± 0.1	0.2 ± ‡
Spleen	26 0 ± 2 8	24 0 ± 2 0	0 9 ± 0 1	13 1 ± 2.3	3.8 ± 1 1	2 7 ± 0[illegible]
Thymus	0 1 ± ‡	0 1 ± ‡	–§	–	–	–
Small intestine	1.3 ± 0 3	0 9 ± 0 1	0 9 ± 0 2	1 1 ± 0.2	0 6 ± 0 1	0 3 ± 0[illegible]
Lungs	2 2 ± 0 6	0.3 ± 0 1	3 1 ± 0.8	2.6 ± 0.2	2.3 ± 0.6	0 6 ± 0[illegible]
Liver	15.8 ± 2.5	16 1 ± 2 0	20 1 ± 3 0	34 2 ± 4.5	33 1 ± 4.5	31.0 ± 5[illegible]
Total recovery	49.2 ± 4.5	45.3 ± 3.2	25 1 ± 2 8	51 1 ± 3 7	40.0 ± 3.5	34.8 ± 5[illegible]

* Hours after i v injection of cells.
§ Minute amounts recovered.
‡ Less than 0 1

injected animals (Table 2a) or by nylon wool filtration of lymph node cells (Table 2b) showed similar distribution patterns. Ecotaxis to the spleen was not distinctly different from that of the thymocytes while accumulation in the lymph nodes was five to ten times as high Radioactive uptake in the other organs tested was similar to that found with thymocytes

The distribution of B-cells in all organs was similar to that of thymocytes (Table 3)

With the exception of a decrease in the amount of labelling found in the lungs, no distinct difference in distribution pattern was found at 24 hours after cell transfer as compared to 4 hours.

Specificity in the ecotaxic patterns was found by *autoradiography* which showed that thymocytes and T-cells localized in the para cortical areas of lymph nodes and in the peri arteriolar areas of the Malphigian bodies of the spleen, while B-cells were found in the outer cortex and medullary sinus of the lymph nodes and in the outer part of the white pulp of the spleen No distinct difference was found in the location of the three cell types in Peyer's patches in the small intestine and none or a few labelled cells were found in the liver and none in the lungs or thymus.

Heat killed cells Ecotaxis of thymocytes (Table 1) T (Table 2a & b) and B-cells (Table 3) were similarly affected (Fig 1) The accumulation in lymph nodes and spleen was almost abolished In contrast, no definite effect was found in the other organs compared to controls. No labelled cells were found in the lymph nodes and spleen on autoradiography The difference at 4 hours and 24 hours was as for control cells.

Protease treated cells Protease influenced the ecotaxis of all cell types similarly (Table 1 2a & b 3 Fig 1) Ecotaxis to lymph nodes was extensively reduced similar to the effect of heat killing On the other hand only a minor reduction in localization to spleen was found There was a tendency to an increase in liver uptake otherwise localization to the rest of the organs tested did not seem to be changed

Autoradiography showed a few cells in the lymph nodes with no change in the internal node distribution as compared to controls. No effect was found on the intrasplenic distribution either

Neuraminidase treated cells Treatment with neuraminidase also seemed to change the ecotaxis of all tested cell types in a similar way (Table 1 2a & b 3 Fig 1)

Lymph node ecotaxis was profoundly re-

TABLE 3. Distribution Pattern of Cr-labelled B-cells from Spleens of Adult Thymectomised Lethally Irradiat d and Bone Marrow Reconstituted Animals T eat d th Prot ase or Neu aminidase at 4 and 24 Hours after i.v. I jection. Recover d Amount Gi en as Per entage of Radioactivity I jected ± Standard Deviation

Organs	Control		Heat killed		Protease treated		Neuraminidase treated	
	4h	24h	4h	4h	4h	24h	4h	24h
Lymph nodes	0.3 ± §	0.3 ± §	0.1 ± §	0.1 ± §	0.1 ± §	0.1 ± §	0.1 ± §	0.1 ± §
Spleen	16.2 ± 2.7	14.4 ± 2.8	1.0 ± 0.1	1.1 ± 0.1	11.6 ± 1.9	12.8 ± 1.7	7.6 ± 1.2	6.3 ± 0.3
Thymus	‡	–	–	–	–	–	–	–
Small intestine	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.3 ± §
Lungs	5.1 ± 0.1	0.7 ± 0.1	5.0 ± 1.0	1.3 ± 0.1	2.7 ± 0.9	0.3 ± §	4.3 ± 0.5	0.8 ± §
Liver	16.6 ± 2.8	15.1 ± 2.3	13.5 ± 2.1	21.8 ± 2.0	27.8 ± 3.7	29.9 ± 1.9	26.3 ± 3.0	26.0 ± 3.7
Total recovery	38.9 ± 1.8	31.1 ± 0.4	20.4 ± 1.1	24.7 ± 1.6	42.9 ± 4.3	43.3 ± 3.7	38.8 ± 2.1	33.7 ± 4.4

Hours after i.v. injection of cells. ‡ Minute amou ts recovered. § Less than 0.1

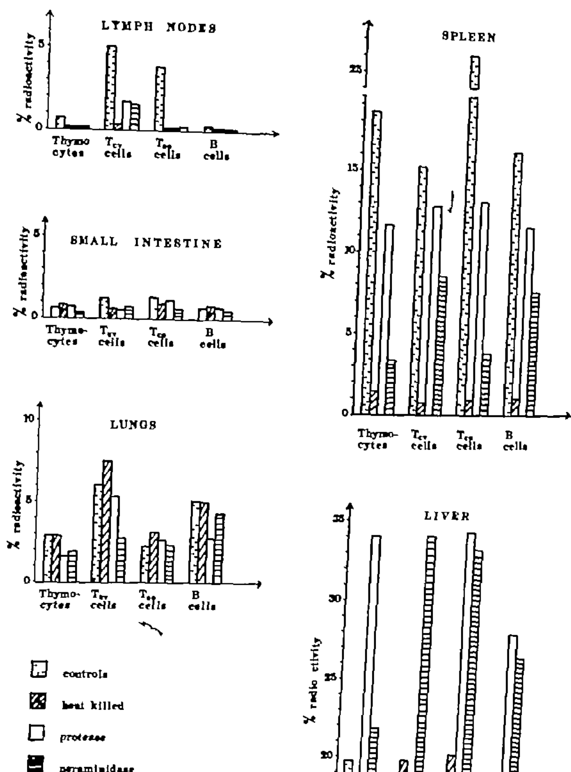


Fig. 1 Histograms of radioactivity recovered from different organs of mice sacrificed 4 hours after i injection of ^{51}Cr labelled thymocytes, T-cells from the spleen of cyclophosphamide-injected animals (Tcy-cells) T-cells from lymph node cells fractionated on a nylon wool column (Tco-cells) and B-cells from the spleen of adult thymectomized, lethally irradiated and bone marrow reconstituted animals, after various forms of treatment

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This treatment too increased the liver uptake, but had no definite effect on the other organs.

The few labelled cells found in lymph nodes and spleen on autoradiography did not show any change in internal localization as compared to controls.

DISCUSSION

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With the exception of an initial trapping in the lungs there was no difference in ecotaxis after 4 or 24 hours. It is unlikely that this is due to cell death as suspensions with more than 20 per cent dead cells were discarded and extensive cell death was consistently accompanied by minimal recovery from both lymph nodes and spleen. The lack of difference might be due to a block in the circulation to the lymph nodes, or rapid circulation caused by rather extensive prewarming of the animals prior to the injection. The distribution of the cells to the expected areas in the lymphoid organs, however, suggests that the circulatory route was via the ordinary pathways, thereby involving the lymphocyte class specific mechanisms (9).

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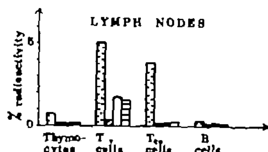
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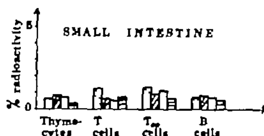
Neuraminidase treatment reduced the uptake both in lymph nodes and spleen in accordance with previous studies (2, 4, 12). However, it has been reported that a similar reduction in B-cell areas was not found following inoculation of neuraminidase containing Newcastle disease virus into mice and rats (22).

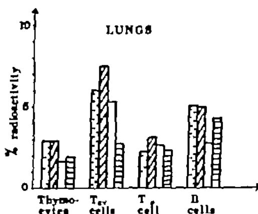
The change in ecotaxis due to enzymatic treatment was less than the effect of heat killing, suggesting that the enzymatic effect is due to a change in the cell membrane structure and does not involve cell viability directly. A possible *in vivo* effect of enzyme injected with the cell suspensions seems to be unlikely in view of previous studies (21). The lack of normalization of ecotaxis in enzyme treated lymphoid cells 24 hours after cell transfer contrasts to previous studies (2, 21). This discrepancy may have arisen because the enzymatic treatment performed in the present study was considerably more extensive.

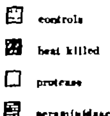
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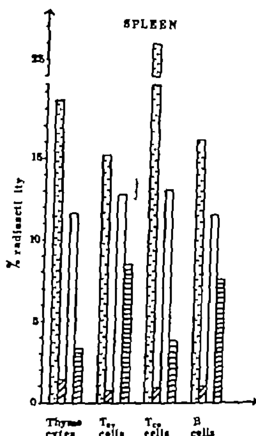
Enhanced susceptibility of the cells to trap-











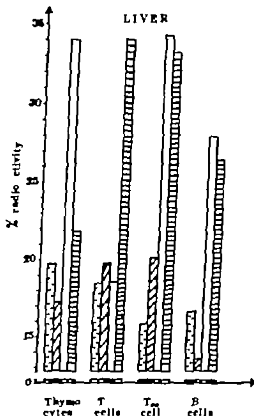


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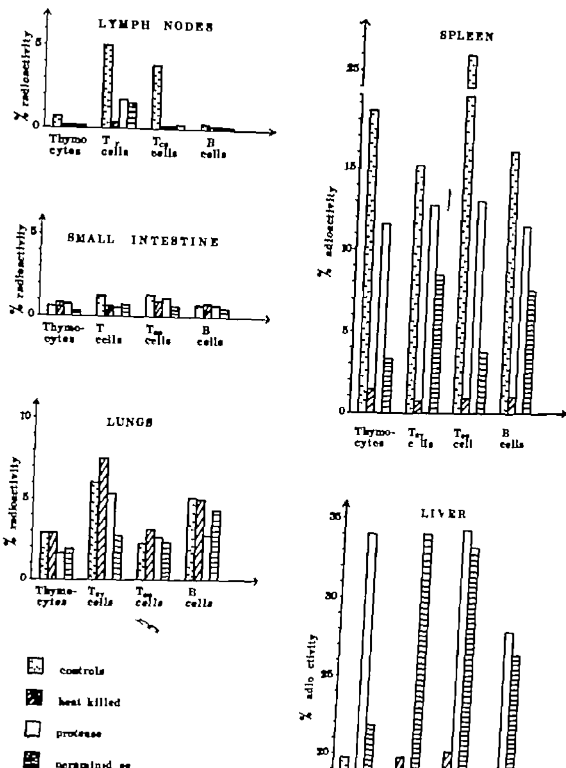


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ping in the liver might prevent them from reaching the lymphoid organs. The rather selective effect of protease treatment on ecotaxis to the lymph nodes makes this assumption unlikely although it cannot be ruled out in the case of neuraminidase treated cells.

In contrast to previous results (2) heat killing did not increase the uptake of isotope in the liver This may be due to the more extensive treatment used for the heat killing of the cells.

It has previously been shown that protease and neuraminidase treatment will reduce the net surface charge of thymocytes by 30 per cent and 40 per cent T-cells by 10 per cent and 60 per cent and B-cells doubtfully and 40 per cent respectively (17 18) Thus it seems unlikely that specific ecotaxis can be a simple electro-static phenomenon as has previously been suggested (1 9)

The present effects of enzyme treatment support the view that cell membrane receptors may be of primary importance for ecotaxis (2 20)

The similarity in effect of both protease and neuraminidase treatment on ecotaxis of the different cell types makes it probable that the receptor groups responsible for ecotaxis to lymph nodes, spleen and Peyer's patches on thymocytes, T and B-cells have grossly similar molecular structure. Three kinds of such "receptors" may be involved in ecotaxis to lymphoid organs The structures regulating the circulation to Peyer's patches seem relatively resistant both to protease and neuraminidase treatment Ecotaxis to lymph nodes seems to be determined by protease and neuraminidase sensitive groups. This receptor' may be a complex one consisting both of protease and neuraminidase sensitive molecules or two equally important single receptor groups. In contrast the "receptors of importance of splenic distribution may consist of mainly neuraminidase sensitive groups The neuraminidase sensitive groups will contain sialic acid molecules while the protease sensitive groups are probably neutral protein complexes (17 18) In contrast a recent study on rat thoracic duct cells indicated that both the trypsin and neuraminidase effects were due to removal of sialic acid groups (20)

The T and B-cell interaction which is of importance for optimal antibody response (7) may take place in lymph nodes and spleen On the basis of the results of the present study it is tempting to postulate that microorganisms able to cause proteolytic and neuraminidase effects on the lymphocyte cell membrane may hamper the ecotaxis of lymphocytes to these organs and thereby possibly prevent an optimal immunological defence reaction

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REFERENCES

1 *Barth R F & Single O M* Migratory patterns of technetium 99m-labelled lymphoid cells. I Effects of antilymphocyte serum on the organ distribution of murine thymocytes Cell Immunol *17* 83–95 1975

2 *Berney S N & Gesner B M* The circulatory behaviour of normal and enzyme altered thymocytes in rats. Immunology *18* 681–691 1970

3 *Droege H Malchow D & Strominger J L* Cellular heterogeneity in the thymus II Distribution patterns of different lymphocyte populations in chickens. Eur J Immunol *2* 161 166 1972

4 *Gesner B M Woodruff J J & McCluskey R T* An autoradiographic study of the effect of neuraminidase or trypsin on transfused lymphocytes. Amer J Path *57* 215 230 1969

5 *Haugen A Helgeland S & Grot A* Localization of antigen in thin sections of bacteria by the immuno-peroxidase technique Acta path. microbiol scand Sect. B *83* 79 90 1975

6 *Lance F M & Taube R A* Segregation of lymphocyte population through differential migration. Nature *221* 841 843 1969

7 *Miller J F A P & Mitchell G F* Thymus and antigen-reactive cells. Transplant. Rev *1* 3 42 1969

8 *Parrott D M V Ferguson A & de Sousa,*

M. A. B., Factors affecting the traffic and segregation of lymphoid cell populations. Int. Arch. Allergy 45 240–248, 1973.
9. *Parrott D M V & de S usa, M A B* Thymus dependent and thymus independent populations origin, migratory patterns and lifespan. Clin. exp. Immunol. *8* 663–684 1971
10 *Reff M C.*, Surface antigenic markers for distinguishing T and B-lymphocytes in mice. Transplant. Rev *6* 52–80, 1971
11 *d Sousa M A. B.*, Kinetics of distribution of thymus and marrow cells in the pheripheral lymphoid organs of the mouse ecotaxis. Clin. exp. Immunol. *9* 371–380, 1971
12. *Sprent J* Unpublished results quoted in Circulating B- and T-lymphocytes of the mouse. I. Migratory properties. Cell. Immunol. *7* 9–39 1973
13. *Th nold S Hersund V & Hartvet F* Complement factors and the growth of Ehr lich carcinoma. Acta path. microbiol. scand. Sect B, *81* 373 381 1973
14 *Trizio D & Cudkowicz, G* Separation of T and B lymphocytes by nylon wool columns E aluation of efficacy by functional assays in vo. J Immunol *113* 1093–1097 1974
15. *T k J L & Poulter L. W* Selective depletion of lymphoid tissue by cyclophosphamide. Clin exp Immunol *10* 285–296, 1972.
16 *W g, J V* Electrophoresis of lymphoid cells. Characterization of T and B-lymphocytes and two populations of thymocytes in the mouse. Acta path. microbiol. scand. Sect. A. Suppl. 236 101–111 1973
17 *W g, J N.*, Effect of neuraminidase on lymphoid cells. Difference in structure of B- and T-cells and thymocytes of the mouse shown by cell electrophoresis and sialic acid determination. Scand. J Immunol. 3 357–363 1974
18. *Wäg J V.*, Electrophoresis of lymphoid cells. Differences in the cell membrane structure of murine thymocytes, T and B-cells revealed by enzyme and formalin treatment. Clin. exp. Immunol. 22 91–101 1975
19. *Wäg J N & Thunold S* Electrophoresis of lymphoid cells. Characterization of human B- and T-cells in peripheral lymphoid tissues. Clin. exp Immunol. *15* 497–506 1973
20. *Woodruff J J.*, Role of lymphocyte surface determinants in lymph node homing. Cell. Immunol *13* 378–384 1974
21 *Woodruff J J & Gesner B. M* The effect of neuraminidase on the fate of transfused lymphocytes. J exp Med. *129* 551–567 1969
22. *Woodruff J F & Woodruff J J* Virus-induced alterations of lymphoid tissues. I. Modification of the recirculating pool of small lymphocytes by Newcastle disease irus. Cell Immunol. *1* 333–354 1970.
23 *Zatz M M & Lanc E. M.*, The distribution of chromium 51-labelled lymphoid cells in the mouse. A survey of anatomical compartments. Cell. Immunol. *1* 3–17 1970.

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BRIEF REPORTS

TRANSFER FACTOR, SARCOIDOSIS AND CELLULAR IMMUNITY

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Platz, P., Grob P J Jonsson V Lorenzen, I & Thomsen, M Transfer factor sarcoidosis and cellular immunity Acta path. microbiol scand Sect C 84 68–70 1976

A 27 year-old man with disseminated sarcoidosis was treated with repeated injections of Transfer Factor Grossly abnormal immunological *in vitro* parameters were normalized but in spite of these changes the clinical condition deteriorated.

Key words Transfer factor sarcoidosis cellular immunity

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Transfer Factor (TF) is an ultrafiltrable extract of leucocytes which is claimed to be able to transmit immunological reactivity in man (for review *Hitzig & Grob* (1974)) We report a therapeutical trial with TF in a patient with severe sarcoidosis.

The patient was a 27 year-old man in whom the diagnosis of sarcoidosis was established by microscopic examination of a lymph node biopsy obtained on mediastinoscopy During the first three years of the disease bilateral pulmonary sarcoidosis was the dominating clinical feature The patient then developed aphasia and progressive right hemiplegia reflecting involvement of the central nervous system. EMI scanning revealed a focus in the left internal capsule. Treatment with large doses of prednisone as well as high voltage X ray treatment in the left hemisphere did not influence the progression of the disease Between 11 Nov and 27 Dec. 1974 the patient received a total of 22 ampoules of TF given with 1–3 weeks interval (See Fig 1) For details of TF production and therapy see 3 The treatment did not affect the condition of the patient who ultimately died from pulmonary infection and respiratory insufficiency Autopsy could not be performed.

Skin test with tuberculin was positive in 1961 and became negative in April 1974 *In vitro* lymphocyte transformation was performed with mitogens PHA PWM and specific antigens (*Candida albicans E coli PPD* and *Staph. aureus*) according to the methods described elsewhere (1) Before TF therapy the patient's lymphocytes were completely non reactive to all mitogens and all specific antigens tested

After the first series of TF a marked increase in the responses to PHA and PWM was seen (*Fig 1*) Also the response to specific antigens was increased but only minor changes were observed after the first series Normal response to *Staph aureus, E coli* and *Candida albicans* was seen after the second series of TF (Fig 1)

The percentage of T-cells in peripheral blood was measured by spontaneous SRBC-rosette formation (2, 5) (normal range 51–84 per cent) B-cells by EAC-rosette formation (5 6) or membrane fluorescence (7) normal range 8 28 per cent. The total lymphocyte count was normal and showed only minimal changes during treatment. Before initiation of TF therapy the T-cell count was subnormal (47 per cent) and the number of B-cells increased (41–50 per cent)

After the first series of TF injections, B- as well as T-cell count were normalized and remained so during the observation period (*Fig 2*)

In conclusion it appeared possible with large doses of TF (TF from about 3 litre of blood) to change the grossly abnormal immunological

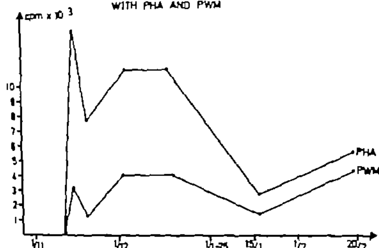
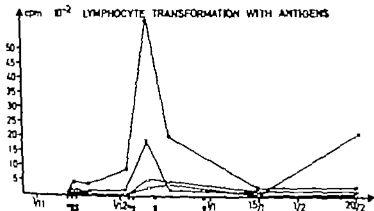


Fig 1 Lymphocyte transformation with mitogens and antigens in a 27-year old man with sarcoidosis treated with repeated injections of Transfer Factor



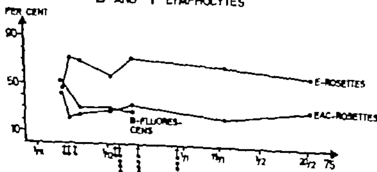


Fig 2 Peripheral T and B-lymphocytes in a 27-year old man with sarcoidosis treated with repeated injections of Transfer Factor

feature to normal reactivities but in spite of these changes the clinical condition deteriorated.

References 1 *Andersen V Thomsen M & Cohn J* Acta path. microbiol scand. Sect. C, *83* 177–183 1975 —2 *Bentwich Z Douglas S D Siegal F P & Kunkel H C* Clin Immun immunopathol *1* 511 1973 —3 *Grob P J Franke C Reymond J F Frei Wettstein M* Europ. J clin. Invest. *5* 33 43 1975 —4 *Hitzig W H & Grob P J* Progr in clinical Immunology *2* 69 100 1974 —5 *Jansson I* Scand J Haemat. *13* 361 369 1974 —6 *Mendes N F Tolnai M E Silveira N P A Gilbertsen R B & Metzger R* J Immunol *111* 860–967 1973 —7 *Siegal F P Pernis B & Kunkel H G* Eur J Immunol. *1* 482–486 1971

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HUMAN COLOSTRAL IgA INTERACTING WITH STAPHYLOCOCCAL PROTEIN A

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Grov, A. Human colostral IgA interacting with staphylococcal protein A. Acta path. microbiol. scand. Sect. C, 84: 71–72, 1976.

Human colostral IgA from three apparently healthy mothers were all divided into protein A reactive and protein A non-reactive fractions on a Sepharose-protein A column. The reactive fractions, giving no direct precipitation but co-precipitations, were found to interact with protein A through the Fc-region.

Key words: Human colostral IgA; staphylococcal protein A.

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Until recently it was supposed that only immunoglobulins of the IgG class interacted with protein A of *Staphylococcus aureus* through the Fc-region (1, 7, 6, 2). However, three of seven IgM proteins from sera of patients with macroglobulinemia as well as three IgM preparations from different pools of normal human sera have now been found to contain protein A reactive fractions (3, 4). These IgM fractions gave no direct precipitation in agar, but became fixed to protein A-Sepharose columns and gave co-precipitation, their primary protein A reactive sites being localized to the Fc-region.

I   iew of this, the earlier reported precipitation between protein A and human colostral IgA (10) has been examined with respect to the interaction mechanism.

Materials and Methods

Immunoglobulin preparations. Samples of human colostrum from three apparently healthy mothers ere obtained from the maternity department of the University hospital. Casein-free milk whey was prepared by acidifying (to pH 4.6 with 1 N HCl) skimmed milk which was subsequently centrifuged to sediment the precipitated casein. The pH of the supernatants were then readjusted to neutrality using 1 N NaOH. Whey samples (4 ml) were gel filtered on   column of Sephadex G-200 (2.5 × 95 cm) in phosphate-buffered saline (PBS) containing 1 M NaCl and 0.02 M NaN_3. The fractions containing IgA as shown by   specific anti-IgA (α) serum were pooled, concentrated by ultrafiltration (Amicon Corp., Mass., USA) and refiltered on the same column. Pooled fractions were then dialysed against PBS, concentrated and checked for purity by immunoelectrophoresis and gar gell diffusion against specific antisera.

Protein concentrations were determined as described in (9). IgG was prepared from pools of normal human and normal rabbit sera (3).

Single hains of olost al IgA were released by reduction with 0.2 M 2-mercaptoethanol (11), the resulting SH groups alkylated in 0.3 M iodoacetamide, and the solution finally dialysed against PBS.

Pepsin-digestion. IgA was digested with pepsin (Sigma, USA) in 0.1 M acetate buffer, pH 4.1, containing 5 mM NaCl t 37 C for 18 h; the enzyme to substrate ratio was 2:100. Tris (0.5 M) was then added to the mixture to give a pH of 8.0.

Immunoadsorbent. Protein A was prepared, coupled to Sepharose 4B (Pharmacia) and used as previously described (2–4).

Antisera. Antisera specific to human α,   and γ heavy chains, λ and κ light chains, and to human Fab fragments were obtained from Hyland, Belgium, anti-secretory component (SC) from Behringwerke AG, Marburg-Lahn, BRD, and antisera specific to IgA_1 and IgA_2 subclasses from Nordic

feature to normal reactivities, but in spite of these changes the clinical condition deteriorated

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Acta path. microbiol. scand. Sect. C, 84 73–76 1976

INTERACTION OF STREPTOCOCCI WITH THE Fc FRAGMENT OF IgG

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Christensen, P Johansson, B. G & Kronvall, G. Interaction of streptococci with the Fc fragment of IgG. Acta path. microbiol. scand. Sect. C, 84 73–76 1976.

The capacity of human IgG to interact with β-haemolytic streptococci was studied in order to localize the site of interaction on the IgG molecule. The reactivity of different proteolytic fragments of IgG with streptococci group A, type M 1 and type M 56, group C and group G was investigated by measuring their inhibitory effect on the uptake of ^{125}I labelled IgG myeloma protein by the streptococci. Equivalent molar amounts of Fc fragment and undigested IgG inhibited the uptake of ^{125}I labelled IgG myeloma protein equally well while only slight inhibition was obtained by F(ab′) preparations. No reactivity was found with IgM, Fab or chymotrypsin produced fragment Fc′ of IgG. The reactivity of IgG with the streptococci was localized to the Fc fragment. Since the Fc′ fragment was non-reactive, the CH2 domain was probably carrying the IgG structures involved in the interaction with streptococci.

Key words: Streptococci; IgG; F(ab′); Fab; Fc; domain; cross reactions.

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An interaction between a surface component of many streptococci and human immunoglobulins, not involving the antibody combining sites has been described (*Kronvall* 1973, *Christensen* 1975, *Christensen & Kronvall* 1974, *Christensen & Oxelius* 1974 and 1975, *Christensen et al.* 1975 a and b, *Christensen & Holm* 1976). Most streptococci tested react with IgG myeloma proteins (group A 97 per cent) but only a few react with IgA myeloma proteins (*Christensen & Oxelius* 1974 and 1975). The probability that the reaction with streptococci involves the antibody combining sites of these myeloma proteins is minimal. The capacity of A, C and G streptococci to absorb ^{125}I labelled IgG myeloma protein is higher than that of B and D streptococci (*Christensen et al.* 1975 a, see also *Christensen & Kronvall* 1974).

This paper concerns the localization on the IgG molecule of the site of interaction with some A, C and G streptococci. The reactivity of the streptococci with different IgG fragments was tested by measuring the capacity of the fragments to inhibit the uptake of ^{125}I labelled intact IgG by streptococci. Using a similar technique, *Kronvall* (1973) detected reactivity in heavy chain preparations but not in light chains, F(ab′) fragments, nor in Fc fragments.

MATERIALS AND METHODS

Streptococcal Strains

Group A streptococcal strains, type M 1 (Colindale No. 8198) and type M 56 (Col.no. 100191)

Immunological Laboratories, Tilburg The Netherlands.

Immunoelectrophoresis was performed using an LKB apparatus, 1 per cent agar Noble (Difco Mich. USA) in barbitone buffer (I 0 025 pH 8 6) and a voltage of 5–6 V/cm.

Serological tests Double diffusion in agar tests for co-precipitation including "star" formation (8) and indirect haemagglutination to detect trace of protein A reactive substances were carried out as before (3 4)

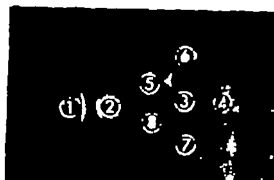


Fig 1 Double diffusion in agar Wells (1) IgA (3 mg/ml) + normal rabbit IgG (NRIgG) (10 mg/ml) (1 1) (2) and (3) protein A (0.5 mg/ml) (4) pepsin-digested IgA + NRIgG (5) IgA (3 mg/ml) (6) and (7) NRIgG (10 mg/ml) (8) pepsin-digested IgA (3 mg/ml)

Results and Discussion

All the three different colostral IgA proteins isolated were found to react with anti-α and anti-SC sera Two of the proteins reacted with both anti-λ and anti κ sera, the third one only with anti κ In contrast to anti IgA_1 anti IgA_2 gave a precipitation line in agar with all preparations. However tanned sheep erythrocytes sensitized with the IgA preparations agglutinated in both anti sera No direct precipitation was observed with protein A in agar gel diffusion, but all the three IgA preparations gave co-precipitation and "star" formation (Fig 1) and when applied to a Sepharose-protein A column they separated into protein A reactive (pA+) and protein A non reactive (pA—) fractions. Only the pA+ fractions were found to give co-precipitation and "star" formation and appeared more homogeneous than the pA— fractions on immunoelectrophoresis. The amounts of pA+ IgA were 20 per cent, 30 per cent and 35 per cent respectively in the three preparations. All fractions (pA+ and pA—) contained SC. Tanned sheep erythrocytes sensitized with either pA+ or pA— fractions agglutinated in both anti-IgA and anti-IgA sera indicating that there is no correlation between IgA subclasses and interaction with protein A. However tanned erythrocytes sensitized with an IgA myeloma protein also agglutinated in both antisera

Pepsin-digested samples of IgA (pA+) were found to retain their reactivity with anti-Fab serum as compared with equal concentrations of non-digested samples The ability to agglutinate normal rabbit erythrocytes was also retained The reactivity with anti-α serum was, however almost completely absent suggesting that most of the Fc region was destroyed by pepsin. None of the pepsin-digested materials became fixed to the Sepharose-protein A column. Nor did they affect co-precipitations, and tanned erythrocytes sensitized with these material did not agglutinate in protein A solutions.

These results indicate that the primary reactive site of these IgA in interaction with protein A is localized to the Fc region

The ability to co-precipitate was lost with reduction and alkylation of IgA (pA+) but heavy chains were still fixed to the immunoadsorbent, indicating that as for IgM (3) structures other than the primary reactive site are necessary to form co-precipitates.

Thus, all the three samples of human colostral IgA contained a protein A reactive fraction, the proportions pA+/pA— being variable. Although the pA+ materials were found to interact with protein A through the Fc-region, trace of Fab-reactivity as suggested previously (10) cannot be excluded. The presence of pA+ fractions has also been demonstrated in serum IgA (unpublished) but the frequency of occurrence is uncertain. Most probably the binding of serum IgA to *Staphylococcus aureus* as observed by *Harboe & Folling* (5) involves the same mechanism as found for colostral IgA.

References 1 *Forsgren A & Sjoquist J* J Immunol 97 822–827 1966 —2 *Grov A* Acta path. microbiol. scand Sect. A suppl 236 77–83, 1973 —3 *Grov A* Acta path. microbiol. scand. Sect. C, 83 173–176 1975 —4 *Grov A* Acta path. microbiol. scand Sect C 83 325–327 1975 —5. *Harboe M & Folling I* Scand. J Immunol 3 471 482 1974 — 6 *Kronvall G Seal U S Finstad J & Williams R C Jr* J Immunol 104 140 147 1970 — 7 *Kronvall G & Williams R C Jr* J Immunol 103 828–833 1969 —8. *Kronvall G & Williams R C Jr* Immunochemistry 8 577–580 1971 —9 *Lowry O H, Rosebrough N J Farr A L & Randall R L.* J biol. Chem. 193 265 275 1951 —10 *McDowell G Grov A & Oeding P* Acta path. microbiol. scand Sect. B 79 801 804 1971 – 11 *Morrison S L. & Koshland M E* Proc. Nat. Acad. Sci. (Wash) 69 124–128 1972

INTERACTION OF STREPTOCOCCI WITH THE Fc FRAGMENT OF IgG

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The capacity of human IgG to interact with β-haemolytic streptococci was studied in order to localize the site of interaction on the IgG molecule. The reactivity of different proteolytic fragments of IgG with streptococci group A, type M 1 and type M 56 group C and group G was investigated by measuring their inhibitory effect on the uptake of [125]I labelled IgG myeloma protein by the streptococci Equivalent molar amounts of Fc fragment and undigested IgG inhibited the uptake of [125]I labelled IgG myeloma protein equally well while only slight inhibition was obtained by F(ab′)₂ preparations. No reactivity was found with IgM Fab or chymotrypsin produced fragment Fc′ of IgG. The reactivity of IgG with the streptococci was localized to the Fc fragment Since the Fc′ fragment was non-reactive, the CH2 domain was probably carrying the IgG structures involved in the interaction with streptococci.

Key words Streptococci IgG F(ab′)₂ Fab Fc′ domain cross reactions.

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An interaction between a surface component of many streptococci and human immunoglobulin, not involving the antibody combining sites has been described (*Kronvall* 1973 *Christensen* 1975 *Christensen & Kronvall* 1974 *Christensen & Oxelius* 1974 and 1975 *Christensen et al* 1975 a and b, *Christensen & Holm* 1976) Most streptococci tested react with IgG myeloma proteins (group A 97 per cent) but only a few react with IgA myeloma proteins (*Christensen & Oxelius* 1974 and 1975) The probability that the reaction with streptococci involves the antibody combining sites of these myeloma proteins is minimal. The capacity of A, C and G streptococci to absorb [125]I labelled IgG myeloma protein is higher than that of B and D streptococci (*Christensen et al.* 1975 a, see also *Christensen & Kronvall* 1974)

This paper concerns the localization on the IgG molecule of the site of interaction with some A, C and G streptococci. The reactivity of the streptococci with different IgG fragments was tested by measuring the capacity of the fragments to inhibit the uptake of [125]I labelled intact IgG by streptococci. Using a similar technique, *Kronvall* (1973) detected reactivity in heavy chain preparations but not in light chains, F(ab′)₂ fragments, nor in Fc fragments.

MATERIALS AND METHODS

Streptococcal Strains

Group A streptococcal strains type M 1 (Colindale No. 8198) and type M 56 (Col.no 100191)

were kindly supplied by the Central Public Health Laboratories, Colindale London. One group C, strain 81 C and one group G strain 115 G were isolated from clinical specimens. Streptococcal grouping was performed as described previously (*Christensen et al.* 1973) The streptococci were cultured in Todd Hewitt broth, harvested and suspended to 2.5 × 10^{10} streptococci/ml phosphate buffered saline (PBS 0 12 M NaCl 0.03 M phosphate pH 7.2) (*Christensen & Oxelius* 1974)

Immunoglobulin Preparations

IgG myeloma protein was purified as described earlier (*Christensen & Oxelius* 1974) Commercial pooled human IgG was purchased from AB Kabi, Stockholm (batch no. 44791) IgG preparations were labelled with ^{125}I using chloramine-T according to *McConahey & Dixon* (1966)

IgM was isolated by gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals) of a serum pool from 10 apparently healthy human individuals (*Flodin & Killander* 1962) The preparation did not contain any IgA or IgG as demonstrated by immunodiffusion tests (*Ouchterlony* 1949) or electroimmuno assay (*Laurell* 1965)

Polyclonal human IgG used for the preparation of fragments, was obtained by ammonium sulphate fractionation of serum proteins remaining in solution after addition of octanoic acid (final concentration, 8 per cent w/v) to fresh human serum (*Steinbuch & Audran* 1969) Trace amounts of contaminating proteins were removed by passing the protein solution through a DEAE-cellulose column, equilibrated with 0 05 M Tris-HCl pH 7 4

Pepsin digestion of IgG was performed at 37 C for 18 h at an enzyme substrate ratio of 1 100 as described by *Turner et al.* (1970) F(ab)$_2$ fragments were isolated by gel filtration on Sephadex G-200 superfine (*Ekman et al.* 1975)

Papain degradation of IgG was performed at 37 C for 5 h in the presence of cysteine (enzyme substrate ratio 1 100) mainly as described by *Gergely et al.* (1967) After gel chromatography on Sephadex G-100 the material containing Fab and Fc fragments was further fractionated by ion exchange chromatography on DEAE-Sephadex A 50 for the separation of these two fragments.

Chymotryptic degradation of IgG was performed as described by *Gerrard et al.* (1974) The chFc fragment was isolated by gel chromatography on Sephadex G-100 superfine elutriated (*Ekman et al.* 1975)

The purity of all fragments obtained were tested by agarose gel electrophoresis (*Johansson* 1972) immunoelectrophoresis against rabbit anti-human IgG and with SDS-polyacrylamide gel electrophoresis (*Gerrard & Bonner* 1974) without the presence of reducing agents. Except for a minor contamination of Fab fragments (5 per cent) in the Fc preparation detected in immunoelectrophoresis, no impurities were revealed by these methods. The F(ab)$_2$ fragment preparation was judged to contain less than 5 per cent undigested IgG. Protein concentrations were measured using a modification of Folin s method (*Lowry et al* 1951)

Determination of the Uptake of ^{125}I Labelled IgG by Streptococci Inhibition Experiments with Immunoglobulins and IgG Fragments

The uptake of ^{125}I labelled IgG myeloma protein by streptococci was measured as described earlier (*Christensen & Oxelius* 1974) The reactivity of IgG fragments with streptococci was determined by their capacity to inhibit the uptake of ^{125}I labelled IgG by the streptococci. This inhibitory capacity was compared with that of unlabelled undigested immunoglobulin. Preparations of fragments or undigested IgG were mixed with 1 µg of radio-labelled IgG and 0.2 ml of a standard suspension of the streptococci was then added. The uptake of ^{125}I labelled IgG was measured after centrifugation and the uptake expressed in per cent of 1 µg labelled IgG added. For comparisons, IgG fragment concentrations were expressed as the weight equivalents of undigested IgG containing the same molar amount of the fragment hence 1 µg IgG equivalents of F(ab)$_2$, Fab, Fc and Fc correspond to 0 67 0.33 0.33 and 0 17 µg of these fragments, respectively

Fractions obtained by ion exchange chromatography of 0 7 g papain digested IgG on a DEAE A 50 column were used in inhibition experiments 100 µl of each fraction was mixed with 1 µg ^{125}I labelled IgG myeloma protein and the uptake of radioactivity by streptococci in 0.2 ml volumes of the standard suspension measured.

RESULTS

Localization on IgG of the Site of Interaction with Streptococci not Involving the Antibody Combining Sites

The inhibition of the uptake of ^{125}I labelled IgG myeloma protein by *Streptococcus pyogenes* group A, type M 1 by some IgG fragments was compared with the inhibiting effect of undigested IgG and IgM (Fig 1) The uptake was inhibited equally well by Fc fragments, IgG myeloma protein and pooled human IgG Human IgM did not inhibit the uptake of ^{125}I labelled IgG while a slight inhibition was seen by F(ab)$_2$ fragments. Similar results were obtained with streptococcal strains M 56 and 81 C or by the use of

^{125}I labelled pooled human IgG instead of labelled IgG myeloma protein.

Fractions obtained by ion exchange chromatography of papain-digested IgG were used in inhibition experiments only the peak containing Fc-fragments (peak II) could inhibit the uptake of ^{125}I labelled IgG myeloma protein by group A streptococci, type M 1 or M 56 (Fig 2)

No inhibition was obtained with chFc 15 µg (corresponding to 90 µg undigested IgG) of the uptake of ^{125}I labelled IgG myeloma protein by streptococcus group A, type M 1 type M 56 or group C, strain 81 C and group G strain 113 G

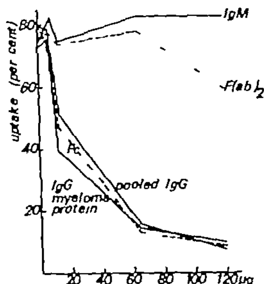


Fig 1 Inhibition of the uptake of ^{125}I labelled IgG myeloma protein (1 µg added) on group A, type M 1 streptococci by different immunoglobulins and IgG-fragments. Abcissa amount of fragments or immunoglobulins added declared in equivalents of undigested IgG 1 µg undigested IgG equivalents of $F(ab)_2$, Fc and IgM correspond to 0.67 0.33, and 5 µg of these preparations. Ordinate uptake of 1 µg ^{125}I labelled IgG added.

DISCUSSION

The present investigations have shown that equivalent amounts of Fc-fragments and undigested IgG inhibit the uptake of ^{125}I labelled IgG myeloma protein by three different streptococcal strains equally well, while only a slight inhibition was obtained with F(ab) preparations. The latter inhibition might be caused by smaller amounts of undigested IgG present in the F(ab) preparations. Thus, the interaction between streptococci and IgG not involving the antibody combining sites, is taking part between the Fc fragment of IgG and the streptococci. In his description of the interaction, *A onsell* (1973) found no reaction with the Fc fragment. These contradictory results could be due to differences in the length of time of digestion with papain and a resulting digestion of the reactive sites on the Fc-fragment.

No reactivity of streptococci with Fc fragment preparations was found, indicating that the CH2 domain might be involved in the interaction. Investigations are in progress to elucidate this possibility

Several immunological cross-reactions between streptococci and human tissues have been described (*Zabriskie* 1967 *Kaplan* 1968 and *G ash g* 1972) None of these studies took into account an Fc-receptor on the streptococci. Due to the presence of such an Fc receptor streptococci of group A, C and G

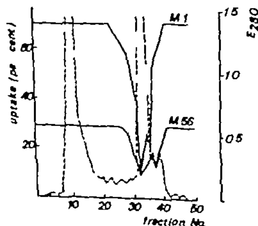


Fig. 2 Inhibition of uptake by streptococci group A, type M 1 and M 56, of ^{125}I labelled IgG myeloma protein by fractions obtained from ion exchange chromatography on DEAE-A 50 of papain digested human IgG. Dotted line extinction at 280 nm of the fractions. Solid line per cent uptake of 1 µg radiolabelled IgG by M 1 streptococci and M 56 streptococci.

were kindly supplied by the Central Public Health Laboratories, Colindale London. One group C, strain 81 C and one group G strain 113 G were isolated from clinical specimens. Streptococcal grouping was performed as described previously (*Christensen et al.* 1973) The streptococci were cultured in Todd Hewitt broth, harvested and suspended to 2.5 × 10^{10} streptococci/ml phosphate buffered saline (PBS 0 12 M NaCl 0.03 M phosphate, pH 7 2) (*Christensen & Oxelius* 1974)

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RAT INTESTINAL GLYCOPROTEIN LOWERING BACTERICIDAL ACTIVITY OF SERUM ON ^{32}P LABELLED *E COLI*

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Wiksnes, R Midtvedt, T & Trippestad, A. Rat intestinal glycoprotein lowering bactericidal activity of serum on ^{32}P-labelled *E. coli*. Acta path microbiol. scand. Sect. C, 84 77–85 1976.

A glycoprotein fraction, by which the ^{32}P-releasing activity of serum on labelled *E. coli* is lowered, was isolated from caecum content of germfree rats. The glycoprotein contained 83 1 per cent carbohydrate 0 8 per cent sulphate, and 16.1 per cent protein. The molecular weight ranged from 3.2×10 to 2.4×10^6 and the iso-electric point from pH 0.9 to 3.0 Physiologically it may play a role in the interaction(s) between the host and its intestinal flora.

Key words Intestinal glycoprotein serum bactericidal activity *E. coli* rat.

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Recent investigations indicate that substances of polysaccharide or glycoprotein nature exerting inhibition of serum bactericidal activity are secreted from the mucosal layer of the intestinal tract of the rat, especially from that of caecum (28) The present study has been performed to gain information about the chemical nature of the substances responsible for this inhibitory effect.

MATERIALS AND METHODS

Reagent

All reagents were of analytical grade unless otherwise stated

Equipment

Absorbance at 280 nm of eluates was determined by LKB 8300 Uvicord II For estimation of carbohydrates, Hewlett Packard, Model 5750 gas chromatograph, fitted with flame ionization detector was used Amino acid analysis was performed using a Bio-Cal BC 200 amino acid analyzer Radioactivity was determined in Packard TRI-CARB scintillation counter Sedimentation equilibrium was performed using Beckman Model E Analytical Ultracentrifuge (AnJ-rotor) A double beam photo-electric scanner was used. For electrofocusing, the LKB 8101 column was applied. The I.r spectrum was determined on a Beckman IR 20 spectrometer by using potassium bromide disc, and the UV spectrum was determined on a Beckman DB spectrometer

General Procedures

The crude extract of caecal content from germfree rats was prepared as described (13 28) but precipitation with ethanol was omitted. Materials were dried by lyophilization, followed by evaporation to constant weight in a vacuum desiccator over phosphoric pentoxide.

Gel Chromatography

The Sephadex G-200 column was eluted with 0.05 M sodium chloride the Sepharose 4B column with 0.05 M sodium phosphate buffer pH 7.0 containing 0.1 M sodium chloride. The DEAE-

(*Christensen et al* 1975 a) will react with human or rabbit IgG, irrespective of the specificity of the antibody combining sites. This interaction might give false postive results in comparisons of bacterial and human antigens and therefore must be reconsidered in such studies.

REFERENCES

Christensen P Agglutinability of some selected streptococci by immune complexes. Acta path microbiol. scand Sect. C, *83* 28–34 1975

Christensen P & Holm S E Purification of immunoglobulin G Fc reactive factor from *Streptococcus agalactiae* Acta path microbiol scand. Sect. C, in press.

Christensen P Kahlmeter G Jonsson S & Kronvall G New method for the serological grouping of streptococci with specific antibodies adsorbed to protein A-containing staphylococci Inf Immun. *7* 881–885 1973

Christensen P & Kronvall G Capacity of some group A, B C D and G streptococci to agglutinate sensitized sheep red cells. Acta path. microbiol scand. Sect B, *82* 19–24 1974

Christensen P & Oxelius V A Quantitation of the uptake of human IgG by some streptococci groups A B C and G Acta path microbiol scand Sect. B *82* 475–483 1974

Christensen P & Oxelius V A A reaction between some streptococci and IgA myeloma proteins. Acta path. microbiol scand. Sect. C, *83* *184 188 1975*

Christensen P Oxelius V A & Hoier Madsen M The mechanism of the I agglutination of streptococci by sera from patients with rheumatoid arthritis. Acta path. microbiol. scand. Sect C, *83* 370–376 1975 (a)

Christensen P Soltesz L. V & Oxelius V A Visualization of the binding of IgG myeloma proteins to some group A streptococci. Acta path. microbiol scand. Sect C, in press.

Ekman R Johansson B G & Ravnskov U Gel chromatography on Sephadex gel with narrow particle size distribution obtained on dry elutriation. Anal. Biochem. In press.

Flodin P & Killander J Fractionation of human serum proteins by gel filtration. Biochem biophys. Acta (Amst.) *63* 403–410 1962

Garrard W T & Bonner J Changes in chromatin proteins during liver regeneration. J Biol. Chem. *249* 5570 5579 1974

Gergely J Stanworth D R., Jefferis R Normansell D E. Henney C S & Pardoe G I Structural studies of immunoglobulins. I The role of cysteine in papain hydrolysis. Immunochemistry *4* *101–111 1967*

Gerrard J Komropoulos A & Turner M W Mild chymotryptic clevage of human IgG and its major subclasses. Scand. J Immunol *3* 11–19 1974

Ginsburg I Mechanisms of cell and tissue injury induced by group A streptococci relation to poststreptococcal sequelae J Infect. Dis. *126* 419–456 1972

Johansson B G Agarose gel electrophoresis Scand J Clin Lab. Invest. Suppl. 124 7 15, 1972

Kaplan M H Multiple nature of the cross-reactive relationship between antigens of group A streptococcus and mammalian tissue In Caravano, R. (Ed.) Current research on group *A streptococcus. Excerpta Medica Foundation.* Amsterdam, New York, London, Paris, Milan, Tokyo Buenos Aires 1968 p 139 144

Kronvall G A surface component in group A, C and G streptococci with non-immune reactivity for immunoglobulin G J Immunol. *111* 1401 1406 1973

Laurell C B Antigen antibody crossed electrophoresis. Anal Biochem. *10* 358–361 1965.

Lowry O H Rosebrough N J Farr L A & Randall R. J Protein measurement with the Folin phenol reagent. J Biol Chem. *193* 265–275 1951

McConahey P J & Dixon F J A method of trace iodination of proteins for immunologic studies. Int. Arch. Allergy *29* 185–189 1966.

Ouchterlony O Antigen-antibody reactions in gels Acta path. microbiol. scand. *26* 507–524 1949

Steinbuch M & Audran R The isolation of IgG from mammalian sera with the aid of caprylic acid. Arch. Biochem *134* 279–284 1969

Turner M W Bennich H & Natvig J B Pepsin digestions of human G-myeloma proteins of different subclasses. I The characteristic features of pepsin cleavage as a function of time Clin. Exp. Immunol *7* 603–625 1970.

Zabriskie J B Mimetic relationships between group A streptococci and mammalian tissues Adv Immunol *7* 147–188 1967

Acta path. microbiol. scand. Sect. C, 84 77–85 1976

RAT INTESTINAL GLYCOPROTEIN LOWERING BACTERICIDAL ACTIVITY OF SERUM ON ^{32}P LABELLED *E COLI*

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Wiesner, R., Midtvedt, T & Trippestad, A. Rat intestinal glycoprotein lowering bactericidal activity of serum on ^{32}P-labelled *E coli*. Acta path. microbiol. scand. Sect. C, 84 77–85 1976.

A glycoprotein fraction, by which the ^{32}P-releasing activity of serum on labelled *E. coli* is lowered, was isolated from caecum content of germfree rats. The glycoprotein contained 83.1 per cent carbohydrate 0.8 per cent sulphate, and 16.1 per cent protein. The molecular weight ranged from 3.2×10^5 to 2.4×10^6 and the iso-electric point from pH 0.9 to 3.0. Physiologically it may play a role in the interaction( ) between the host and its intestinal flora.

Key words Intestinal glycoprotein serum bactericidal activity *E. coli* rat.

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Recent investigations indicate that substances of polysaccharide or glycoprotein nature exerting inhibition of serum bactericidal activity are secreted from the mucosal layer of the intestinal tract of the rat, especially from that of caecum (28) The present study has been performed to gain information about the chemical nature of the substances responsible for this inhibitory effect.

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade unless otherwise stated

Equipment

Absorbance at 280 nm of eluates was determined by LKB 8300 Uvicord II For estimation of carbohydrates, Hewlett Packard, Model 5750, gas chromatograph, fitted with a flame ionisation detector was used Amino acid analysis was performed using Bio-Cal BC 200 amino acid analyser Radioactivity was determined in a Packard TRI-CARB scintillation counter Sedimentation equilibrium was performed using a Beckman Model E Analytical Ultracentrifuge (AnJ-rotor) A double beam photo-electric scanner was used. For electrofocusing, the LKB 8101 column was applied. The i r spectrum was determined on Beckman IR 20 spectrometer by using a potassium bromide disc, and the UV spectrum was determined on Beckman DB spectrometer

General Procedures

The crude extract of caecal content from germfree rats was prepared as described (13 28) but precipitation with ethanol was omitted. Materials were dried by lyophilization, followed by evaporation to constant weight in a vacuum desiccator over phosphoric pentoxide.

Column Chromatography

The Sephadex G-200 column was eluted with 0 05 M sodium chloride the Sepharose 4B column with 0.05 M sodium phosphate buffer pH 7.0, containing 0.1 M sodium chloride. The DEAE-

Sephadex (A 50) column was eluted with a linear gradient of sodium chloride (O-1 M) in 0 05 M sodium phosphate buffer pH 7 0 Flow rates for gel filtration were 3 6 ml/cm²/hour for ion exchange chromatography 8 ml/cm²/hour Fractions of 5 ml were collected.

Analytical Procedures

The elution of carbohydrates from columns was monitored by the phenol-sulphuric acid assay (2) by the Gatt & Berman modification of the Elson-Morgan method (hexosamines) (4) and by the periodate-resorcinol assay (sialic acid) (10) The elution of protein was estimated by absorbance at 280 nm or by the Folin-Lowry method (11) Lipopolysaccharides were detected according to the method described in ref 9 sulphate according to ref 20 and phosphate according to ref 3

Incubation with Ribonuclease

The material recovered in fraction D (Fig 4) was incubated with salt free and protease-free bovine pancreatic ribonuclease (Koch-Light Labs. Ltd) activity 70 Kunitz units/mg Ribonuclease (0 1 mg/1 ml) dissolved in 0.5 M Tris HCl buffer pH 7 2 containing 0 1 M sodium chloride, was added to a solution of fraction D (65 mg/2 ml) in the same buffer and incubated at 37 C for 2 hours. The glycoprotein was thereafter fractionated on a column of Sephadex G-100 (48 × 1.5 cm) equilibrated and eluted with 0 05 M Tris-HCl buffer pH 7.2 containing 0 1 M sodium chloride and 8 M urea. The front running glycoprotein, called the purified glycoprotein (PGP) was tested for effect on serum bactericidal activity (Table 1)

Gel Electrophoresis on Agarose Columns

The preparation of 1 per cent agarose gels closely followed the method described by *Holden et al* (5 6) Each gel, containing 250 μg glycoprotein, was stained for carbohydrate (5 24 31) sulphate (12) and for protein, using amido black.

Digestion with Pronase

was carried out at 30 C for 24 hours (28)

Amino Acid Content

was determined by ion exchange chromatography as described in ref 29

Gas Liquid Chromatography

Separation of the trimethylsilyl ethers of the methyl glycosides was performed on a coiled glass column (180 × 0 18 cm) containing 3 per cent Se-30 (Wilkins Instrument & Research Inc.) on Varaport, 100/120 mesh (Varian Aerograph)

Preparation of samples was performed according to a modification (15) of the method of *Clamp et al* (1) except that hydrolysis was performed with 1 M hydrochloric acid in anhydrous methanol at 80 C for 24 hours under nitrogen. Mesoinositol was used as an internal standard.

Molecular Weight

Sedimentation equilibrium was performed at 3000 rev/min at 20 C. A solution of PGP (0.3 mg/0 1 ml) in 0 1 M sodium chloride was used. Partial specific volume was calculated on the basis of the composition of the amino acids and carbohydrates as described in ref 17

Isoelectric Focusing

Electrofocusing of PGP in a pH gradient of 1-6, was performed according to a modification (14) of the method of *Vesterberg & Svensson* (25) One ml of fractions was dialysed against Krebs-Ringer buffer containing 10 mM glucose (16) and thereafter tested for effect on serum bactericidal activity (Fig 5)

Assay for Blood Group Substances

Solutions of PGP (1 mg/ml) from 6 individual rats in 0.9 per cent sodium chloride, were used in haemagglutination inhibition assays with anti-A/A_1 cells, anti-B/B_1 cells, anti H(human)/O cells, anti H (Ulex Europaeus)/O cells, anti-Le^a/Le (a+)cells, anti Le^{bL}/Le(b+)cells, and anti-O (H)/O cells.

Incubation with Neuraminidase

PGP (15 mg) was incubated with neuraminidase (20 U calc for N acetylneuraminyllactose) from *Cl. perfringens* (type V Sigma) in 4 ml 0.1 M acetate buffer pH 5 0 for 24 hours at 37 C. The resulting material, eluted with the void volume on a column of Sephadex G-200 was subjected to analyses for carbohydrates and effect on serum bactericidal activity

Alkaline Borohydride Degradation

The *Iyer & Carlson* technique (8) using 10 mg PGP/ml, has been used.

Inhibition of Serum Bactericidal Activity

Experimental details have been described previously (22, 28) Concentration of sample in the final test suspensions was 0 05 mg/ml if not otherwise stated.

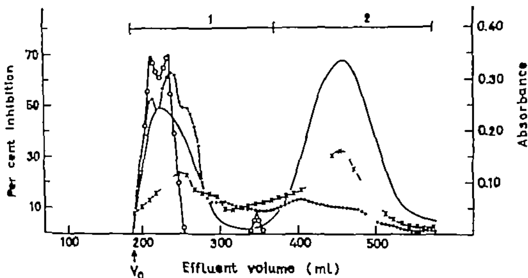


Fig 1 Gel filtration of the crude extract on Sephadex G-200. Absorbance at 280 nm of the eluate was determined (——). Fractions were subjected to the Folin-Lowry test for protein (–x–), the phenol-sulphuric acid test for carbohydrates (●-●-●-) and to tests for inhibition of serum bactericidal activity (O-O-O-)

RESULTS

Relationship between Mucin Concentration and Inhibitory Effect on Serum Bactericidal Activity

Dose response tests showed a linear relationship between the logarithm of the concentration of material of fraction I (Fig 2) and per cent inhibition of bactericidal activity in the range from 0.04 mg/ml to 0.30 mg/ml in final test suspensions (Fig. 3). Whenever a concentration of 0.30 mg/ml was used, 100 per cent inhibition was obtained.

Purified Glycoprotein

Only the peak, first eluted from the Sephadex G-200 column (Fig. 1) contained substances exerting inhibition of serum bactericidal activity (Table 1). The active material was further purified on a Sepharose 4B column. As fraction II (Fig. 2) was less active than fraction I and contained impurities not easily removed, fraction I was chosen for further studies. Ion exchange chromatography of fraction I gave a main fraction, D (Fig. 4) constituting the essential part of the inhibitory

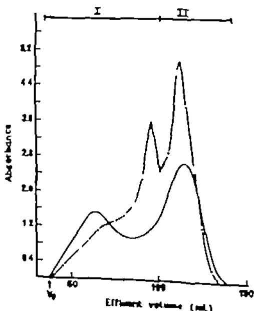


Fig 2 Gel filtration of the front-running material from the Sephadex G-200 column (fraction 1 Fig. 1) on Sepharose 4B. Absorbance at 280 nm of the eluate was determined (——). Aliquots were scanned by the phenol-sulphuric acid assay (●-●-●)

Sephadex (A 50) column was eluted with a linear gradient of sodium chloride (O-1 M) in 0 05 M sodium phosphate buffer pH 7 0 Flow rates for gel filtration were 3 6 ml/cm²/hour for ion exchange chromatography 8 ml/cm²/hour Fractions of 5 ml were collected

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The preparation of 1 per cent agarose gels closely followed the method described by *Holden et al* (5 6) Each gel containing 250 µg glycoprotein, was stained for carbohydrate (5 24 31) sulphate (12) and for protein, using amido black

Digestion with Pronase

was carried out at 50 C for 24 hours (28)

Amino Acid Content

was determined by ion exchange chromatography as described in ref. 29

Gas Liquid Chromatography

Separation of the trimethylsilyl ethers of the methyl glycosides was performed on a coiled glass column (*180 × 0 18 cm*) containing 3 per cent Se-30 (Wilkins Instrument & Research, Inc.) on Varaport, 100/120 mesh (Varian Aerograph)

Preparation of samples was performed according to a modification (15) of the method of *Clamp et al.* (1), except that hydrolysis was performed with 1 M hydrochloric acid in anhydrous methanol at 80 C for 24 hours under nitrogen. Mesoinositol was used as an internal standard.

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Solutions of PGP (1 mg/ml) from 6 individual rats in 0 9 per cent sodium chloride, were used in haemagglutination inhibition assays with anti-A/A_1 cells, anti-B/B_1 cells, anti-H(human)/O cells, anti H (Ulex Europaeus)/O cells, anti-Le^a/Le (a+)cells, anti-Le^{bL}/Le(b+)cells, and anti-O (H^c)/O cells.

Incubation with Neuraminidase

PGP (15 mg) was incubated with neuraminidase (20 U calc. for N acetylneuraminyllactose) from *Cl. perfringens* (type V Sigma) in 4 ml 0.1 M acetate buffer pH 5 0 for 24 hours at 37 C. The resulting material eluted with the void volume on a column of Sephadex G-200 was subjected to analyses for carbohydrates and effect on serum bactericidal activity

Alkaline Borohydride Degradation

The *Iyer & Carlson* technique (8) using 10 mg PGP/ml, has been used.

Inhibition of Serum Bactericidal Activity

Experimental details have been described previously (22 28) Concentration of sample in the final test suspensions was 0.05 mg/ml if not otherwise stated.

TABLE 1 *Inhibition of Bactericidal Activity Exerted by Fractions of Caecal Contents*

Fraction	Inhibition* (%) A	Protein (μg) B	A/B × 10	Yield§ (g)
Crude extract	43	18	24	1.7
Sephadex G-200				
Fraction 1	43	12	38	1.2
Fraction 2	0			
Fraction 1 on Sepharose 4B				
Fraction I	56	10	56	0.7
Fraction II	44	16	28	0.5
Fraction I on DEAE-Sephadex				
Fraction D	59	9	66	0.3
Fraction O	36	9	40	–
RNase-treated fraction D†	60	8	75	0.4

* 50 μg dry weight material in test.
§ From 4 rats.
† Denoted purified glycoprotein (PGP) in text.

in the pH gradient between 0.9 and 3.0. Glycoproteins with iso-electric point within this range seem to exert inhibition of serum bactericidal activity to about the same extent (Fig. 5).

Blood Group Activity

PGP from six individual rats showed haemagglutination inhibition of anti-A, anti-H, and a weak inhibition of anti-B. Inhibition of anti-Le^a, anti-Le^{AL} and anti-O(H^c) could not be demonstrated.

Partly Removal of Sialic Acid

Incubation with neuraminidase reduced the sialic acid content of PGP from 15.33 per cent to 6.91 per cent, and the content of fucose from 8.89 per cent to 3.92 per cent. The amount of the other sugars remained unaltered. Treatment with neuraminidase had no influence upon the inhibitory activity.

Reduced Oligosaccharides

The dialysable reduced oligosaccharides, resulting from alkaline borohydride degradation, had no inhibitory effect on serum bacte-

TABLE 2. *Composition of the Purified Glycoprotein from Rat Caecal Contents*

Components*	Molar proportions (threonine = 1)	% composition§
Fucose	1.05	8.89
Galactose	2.11	19.06
N-Acetylgalactosamine	1.71	20.18
N-Acetylglucosamine	1.60	18.87
N-Acetylneuraminic acid	0.88	15.33
Total carbohydrate	7.35	83.13
Sulphate	0.13	0.75
Aspartic acid	0.03	0.18
Threonine	1.00	5.84
Serine	0.33	2.66
Glutamic acid	0.06	0.48
Proline	0.38	2.17
Glycine	0.26	0.87
Alanine	0.20	0.82
Valine	0.21	1.20
Isoleucine	0.11	0.75
Leucine	0.09	0.81
Arginine	0.06	0.50
Total protein	2.93	16.12

* Traces of lysine and histidine were found.
§ Values are corrected to give a total yield of 100 per cent.

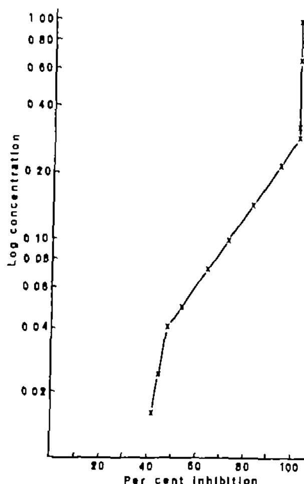


Fig 3 Inhibition of bactericidal activity of rat serum on *E. coli* exerted by fraction I Fig 2

substances (Table 1) Fraction D was removed from the column by 0.3–0.5 M sodium chloride in the buffer solution No inhibitory effect for the material unadsorbed to the column was found or for fraction A B and C in contrast to E, F and G Incubation of D with ribonuclease, followed by gel filtration, resulted in PGP (Table 2) free from ribose as well as arabinose xylose and mannose Treatment with ribonuclease scarcely influenced the inhibitory activity/μg protein (Table 1) Neither ribonucleic acids (brewer's yeast, C F Boehringer & Soehne) nor ribonuclease, treated as PGP had any effect on serum bactericidal activity

Agarose Gel Electrophoresis

Agarose gel electrophoresis of PGP inclusive and exclusive physical de-aggregation and disulphide bond cleaving reagents, revealed a single slowly moving diffuse band, staining for carbohydrate and sulphate. No protein band was detected.

Digestion of PGP with pronase, followed by agarose gel electrophoresis, revealed two bands, staining for carbohydrate and sulphate only Both bands migrated a little faster than the band of undigested glycoprotein

As described earlier (28) treatment with pronase did not reduce the inhibitory activity of the caecal content.

Amino Acids Carbohydrates and Sulphate

PGP gave no absorption peak in the ultra violet region (220-310 nm), using 0.5 mg/ml KRG and a cell depth of 1 cm (Cfr ref 27)

The total amount of amino acids and carbohydrates of PGP are given in Table 2. The figures are the means of the results of three experiments corrected to give a total yield of 100 per cent. Threonine and serine constituted 53 per cent and proline 13 per cent of the total amino acid content of PGP Fucose galactose N-acetylgalactosamine, N acetylglucosamine and sialic acid were the only carbohydrates of PGP

Structural investigations of PGP by infra red analysis gave a spectrum with absorption bands at 3400 cm^{-1} (broad O-H stretch) at 1660 and 1540 cm^{-1} (acetamido group) at 1240 cm^{-1} (S=O stretch in sulphate group) and at 820 cm^{-1} (C-O-S vibration) PGP contained 0 75 per cent sulphate. Content of phosphate could not be demonstrated

Molecular Weight

Sedimentation equilibrium experiments showed a molecular weight range from 8×10^5 to 4×10 for fraction D and 3.2×10^5 to 2.4×10^6 on an average of 6.3×10^5 for PGP

Isoelectric Focusing

When PGP was electrofocused in a pH gradient of 3–10 all of the material migrated to the anode solution. After electrofocusing in a pH gradient of 1-6 for 48 hours, the material applied to the column was observed

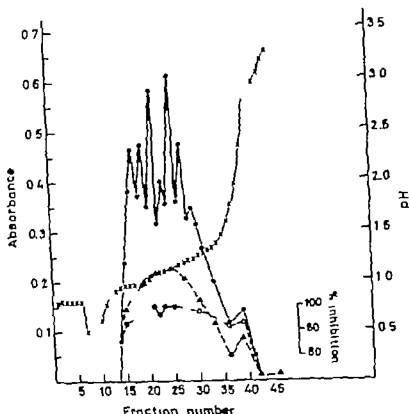


Fig. 3 Electrofocusing of purified glycoprotein from rat caecal contents. The pH of each fraction was determined (x x–x–). Aliquots were subjected to the Folin-Lowry test for protein (▲–▲–▲–), the phenol-sulphuric acid test for carbohydrates (●—●) and to tests for inhibition of serum bactericidal activity of rat serum on *E. coli* (○—○—○)

order to avoid chemical modification of the native mucin macromolecules. Within the limitations of the procedures used, a presence of contaminating proteins or nucleic acids in PGP could not be demonstrated. Purification of active substances resulted in enrichment of sialic acid from 5.0 per cent in the crude extract to 15.3 per cent in PGP. The inhibitory activity of PGP was about three-fold that of the crude extract, while the yield of material was 24 per cent. As inhibitory glycoproteins constitute a major part of the crude extract, a three-fold purification degree seems to be acceptable.

In contrast to a report on a glycopeptide presenting a varying degree of sulphation (7), fraction D with a sulphate content of 0.75 per cent only was adsorbed on DEAE-Sephadex, equilibrated in 0.05 M sodium phosphate buffer pH 7.0. This discrepancy might be explained by the high content of sialic acid, and by the fact that the glycoprotein eluted with the front-running material in the Sepharose 4B column, represents a fraction, tightly bound to the nucleic acid components of the mixture.

PGP contained fucose galactose N-acetylgalactosamine, N acetylglucosamine and sialic acid. A high content of these carbohydrates together with a high content of threonine, serine and proline are characteristic of glycoproteins of the "mucin type" (30). The quantitative relationship between threonine and serine residues was about 2:1 as described for glycoprotein from human gastric aspirations (19).

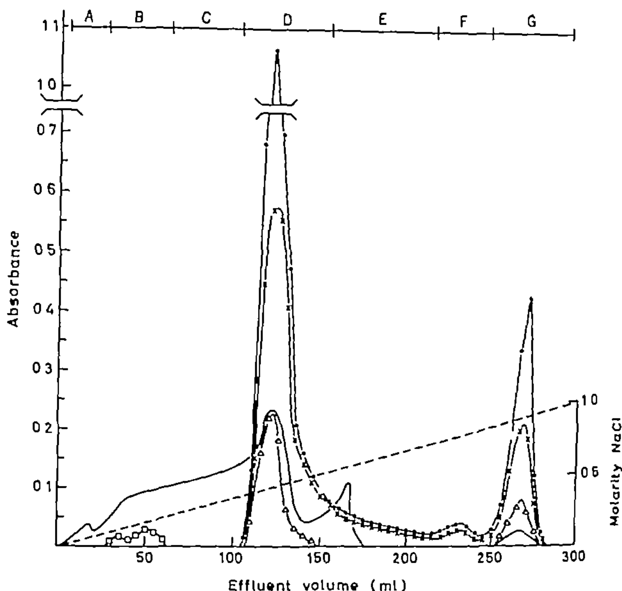


Fig 4 Column chromatography of fraction I (Fig 2) on DEAE-Sephadex (A 50) Absorbance at 280 nm of the eluate was determined (——) Aliquots of fractions were subjected to the phenol-sulphuric acid assay for carbohydrates (●-●-●-) assay for hexosamines (-×-×-×) sialic acid (Δ-Δ-Δ-) and lipopolysaccharides (□-□-□-)

ricidal activity The undialysable material consisting of protein and residual carbohydrates had retained 30 per cent of the inhibitory activity of PGP

Monosaccharides Tested for Inhibition of Serum Bactericidal Activity

Fucose, galactose N-acetylgalactosamine, N acetylglucosamine and N-acetylneuraminic acid were not capable of inhibiting serum bactericidal activity whether tested singly or together The concentration of each monosaccharides in the final test suspension was 0 05 mg/ml

DISCUSSION

In the search for substances, capable of inhibiting bactericidal activity of normal rat serum on *E coli* a high molecular weight glycoprotein fraction from caecal contents of germfree rats has been isolated

A purification procedure avoiding the use of peptide hydrolases has been preferred, in

biotic and conventionalized rats on ³²P-labelled E. coli. Acta path. microbiol. scand. Sect. B, 78 1 5 1970.

14 *Anderluser D H Plentner J J & Carlson D M* The purification and properties of the glycoprotein of pig gallbladder bile. Arch Biochem. Biophys. *145* 155–163 1971

15. *Reinhold, V N* Gas-liquid chromatographic analysis of constituent carbohydrates in glycoproteins. In Hirs, C. H. W. & Timasheff S. N. (Eds) Methods in Enzymology Vol. 25 Academic Press, London, 1972, p. 244–249.

16. *Roberts, J & Quastel J H* Particle uptake by polymorphonuclear leucocytes and Ehrlich ascites-carcinoma cells. Biochem. J *89* 150–156, 1963.

17 *Schachman H K* Ultracentrifugation, diffusion, and viscometry In Colowick, S. P & Kaplan, N O. (Eds.) Methods in Enzymology Vol. 4 Academic Press, London, 1957 p. 32 103

18 *Schrager J* The composition and some structural features of the principal gastric glycoprotein. Digestion 2 73–89 1969

19 *Schrager J & Oates M D G* Further observations on the principal glycoprotein of the gastric secretion. Digestion *3* 231 242, 1970

20 *Terho T T & Hartiala K.* Method for determination of the sulfate content of glycosaminoglycans. Anal. Biochem *41* 471–476 1971

21. *Thompson, G R & Trexler P C* Gastrointestinal structure and function in germ-free or gnotobiotic animals. Gut *12* 230–235 1971

22. *Trippestad A & Midtvedt T* Phagocytosis of ³²P-labelled E. coli by rat peritoneal polymorphonuclear leucocytes Evaluation of a method. Acta path. microbiol. scand. *74:* 259–273 1968.

23. *Vesterberg O & Svensson H.* Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradient IV Further studies on the resolving power in connection with separation of myoglobins. Acta Chem. Scand. *20* 820–834 1966.

24 *Wardi A H & Michos G A.* Alcian Blue staining of glycoproteins in acrylamide disc electrophoresis. Anal. Biochem. *49* 607–609 1966

25 *Watkins, W M* Blood group substances. In the ABO system the genes control the arrangement of sugar residues that determines blood-group specificity Science (Wash. D.C.) *152* 172–181 1966.

26 *Watkins W M* Blood-group specific substances In Gottschalk, A. (Ed.) Glycoproteins, vol. 5 Elsevier Press, Amsterdam, 1972, p. 839

27 *Watkins, W M* p. 840 in Glycoproteins, see ref 26

28. *Wrammer R., Trippestad A., Midtvedt T & Wold J K.* Studies of intestinal and faecal substances from germfree rats inhibiting bactericidal activity of rat serum. Acta path. microbiol. scand. Sect. B, *81* 233–241 1973

29 *Wold J K Khan R. & Midtvedt T.* Intestinal glycoproteins of germfree rats. Acta path microbiol. scand. Sect. B 79 525–530, 1971

30 *Yosizawa, Z* Sulphated glycoproteins, p. 1002–1004 in Glycoproteins, see ref. 26.

31 *Zacharius R. M., Zell, T E., Morrison J H & Woodlock, J J* Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. *30* 148–152 1969.

Heterogeneity was demonstrated both by agarose gel electrophoresis, sedimentation equilibrium analyses, iso-electric focusing and haemagglutination inhibition (25) Physical aggregation or presence of cystine bridges could not be demonstrated A molecular weight range like that of PCP has been reported to apply to blood-group substances isolated from ovarian cyst fluid (26)

A number of factors may contribute to the polydispersity of the isolated glycoprotein The caecal mucin is probably contaminated with mucins, originating from other parts of the digestive tract as well as intracellular mucin and nucleic acids from the disintegration of the rapidly dividing (21) and exfoliated epithelial cells of the gut The molecular weight and iso-electric point range may partly be due to a varying extent of completion of the carbohydrate chains (microheterogeneity) and glycosylation of the amino acid acceptors of the peptide chain. Besides, gastro-intestinal secretions may be polydisperse with respect to sulphate and sialic acid content as described for glycoproteins from human gastric aspirates (18) The native macromolecules have most likely been subjected to the influence of proteases and glycosidases in the gastro-intestinal tract.

Experiments with PGP treated with sialidase or pronase, indicate that the charge and size of the glycoprotein molecules is not critical for the exertion of inhibitory activity Alkaline borohydride degradation of PGP indicated the necessity for a presence both of carbohydrates and protein for the inhibitory activity on humoral host defence factors. Such factors are excreted to a varying extent into the intestinal lumen The inhibition found might therefore play a role in the interaction(s) between the host and its intestinal flora

The authors want to thank Dr *R Nordhagen* National Institute of Public Health Oslo for the haemagglutination inhibition assays, Dr *T B. Christensen* Department of Biochemistry University of Oslo for the sedimentation equilibrium analyses, and Dr *K Sletten* Department of Biochemistry University of Oslo for the amino acid analyses.

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REFERENCES

1 *Clamp J R Dawson G & Hough L.* The simultaneous estimation of 6-deoxy-L-galactose (L-fucose) D-mannose D-galactose, 2 acetamido-2-deoxy D-glucose (N-acetyl-D-glucosamine) and *N* acetylneuraminic acid (sialic acid) in glycopeptides and glycoproteins. Biochem. Biophys. Acta *148* 342–349 1967

2. *Dubois M Gilles K A Hamilton J K Rebers P A & Smith F* Colorimetric method for determination of sugars and related substances. Anal. Chem *28* 350 356 1956.

3 *Fiske C H & Subbarow Y* The colorimetric determination of phosphorus. J Biol. Chem. *66* 375–400 1925

4 *Gatt R & Berman E R* A rapid procedure for the estimation of amino sugars on a micro scale. Anal Biochem *15* 167–171 1966.

5 *Holden K G Yim N C F Griggs L J & Weisbach J A.* Gel electrophoresis of mucous glycoproteins. I Effect of gel porosity Biochemistry *10* 3105–3109 1971

6 *Holden K G Yim N C F Griggs L J & Weisbach J A.* Gel electrophoresis of mucous glycoproteins. II Effect of physical deaggregation and disulfide-bond cleavage Biochemistry *10* 3110–3113 1971

7 *How M J Jones J V S & Doyle J* Comparative studies of Lorenzini jelly from two species of elasmobranch. Part I Preparation of glycopeptides. Carbohydr Res. *11* 207–216 1969

8. *Iyer R. N & Carlson D M* Alkaline borohydride degradation of blood group H substance. Arch. Biochem. Biophys. *142* 101 105 1971

9 *Janda J & Work E* A colorimetric estimation of lipopolysaccharides. FEBS Letters *16* 343–345 1971

10 *Jourdian G W Dean L & Roseman S* The sialic acids XI A periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides. J Biol Chem. *246* 430–435 1971

11 *Lowry O H Rosebrough N J Farr A L & Randall R J* Protein measurement with the Folin phenol reagent. J Biol Chem *193* 265–275 1951

12 *McDevitt C A & Muir H* Gel electrophoresis of proteoglycans and glycosaminoglycans on large-pore composite polyacrylamide-agarose gel Anal. Biochem. *44* 612 622 1971

13 *Midtvedt T & Trippestad A* Opsonizing and bactericidal effects of sera from gnoto-

TABLE 1. *Diagnoses, Sex and Age Distribution and the Occurrence of CMV and EBV Antibodies in 94 Patients with Malignant Diseases and in 152 Controls*

Diagnoses	No. of subjects	Females	Males	Mean age (years)	Age range (years)	No. of pts. with CMV antibodies	No. of pts. with EBV antibodies
Hodgkin's disease	21	7	14	40.4	8–68	11 (52%)	19 (90%)
Lymphoproliferative diseases							
Chronic lymphatic leukaemia	6	1	5	62.8	55–70	3 (50%)	6 (100%)
Acute lymphoblastic leukaemia	19	10	9	16.5	4–70	8 (42%)	12 (63%)
Lympho- and reticulosarcoma	13	7	6	49.9	15–70	6 (50%)	11 (85%)
Myelomatosis	5	3	2	55.4	46–71	2 (40%)	2 (40%)
Total	43	21	22	37.3	4–71	19 (45%)	31 (72%)
Myeloproliferative diseases							
Chronic myelocytic leukaemia	2	1	1	47.5	47–48	1	1
Acute myeloblastic leukaemia	28	10	18	44.4	16–82	19 (68%)	25 (89%)
Total	30	11	19	44.6	16–82	20 (67%)	26 (87%)
All patients	94	39	55	40.6	4–82	50 (53%)	76 (81%)
Controls	152*	79	73	37.6	3–76	83 (55%)	109 (72%)

* One serum was anticomplementary and CMV antibodies were not determined.

MATERIAL AND METHODS

Sera

Sera from 94 patients with malignant diseases and from 152 control subjects were investigated for tissue antibodies, CMV antibodies and EBV antibodies.

Sera from patients and controls had been stored at −20 C for up to five years. The diagnoses of the patients as well as the age and sex distribution of patients and controls are shown in Table 1. The patients can be grouped into three main categories: Hodgkin's disease, lymphoproliferative diseases and myeloproliferative diseases (13). The group of patients with lympho-reticulosarcoma comprised six cases of lymphosarcoma, one case of lymphoma gigantofolliculare, four cases of reticulosarcoma and two cases of lympho-reticulosarcoma. The majority of the patients were under treatment with cytotoxic drugs (cyclophosphamide, mercaptopurine, cytosine arabinoside, daunomycin, busulphan, chlorambucil, and amethopterine) and steroid hormones. Twenty-eight patients had been treated with local irradiation within six months prior to the serological investigation. Sixty-five of the patients were only examined once, while 29 patients were examined on two or three occasions at intervals of from one to 28 weeks. All sera from one patient were studied in the same experiment.

The control group comprised 134 healthy blood donors and 18 hospitalized patients. The patients were not suffering from auto-immune infections or hepatic diseases, and sedimentation rates and haemoglobin concentrations in these patients were within normal limits.

Tissue Antibodies

All sera were investigated for smooth-muscle antibodies (SMA), glomerular antibodies (GA), mitochondrial antibodies (MTA), antinuclear antibodies (ANA) and parietal-cell antibodies (PA) by the indirect immunofluorescent method (IIF) as previously described (1, 2). The antigens were 4 μ thick cryostat sections of rat stomach and rat kidney. All sera were initially tested in a dilution of 1:10 with a heterospecific fluorescein-isothiocyanate (FITC)-conjugated antihuman immunoglobulin (Wellcome Research Laboratories). Chessboard titrations were performed with this conjugate and three sera which contained IgG-SMA, IgA-ANA and IgM-SMA. On the basis of these titrations, the conjugate was used in a dilution which could detect antibodies of the IgG, the IgA, and the IgM classes. Sera which were positive, or doubtfully positive, were titrated in doubling dilution starting at 1:10: they were investigated for antibodies of the IgG, IgA, and IgM classes by means of monospecific FITC-conjugated anti-human immunoglobulins (Wellcome Research Laboratories). The monospecific conjugates did not cross-react in gel precipitation. The molar F/P ratios were 2.9 to 4.1 and the conjugates were used in a dilution corresponding to 1–4 units/ml of antibody.

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SMOOTH-MUSCLE ANTIBODIES AND ANTIBODIES TO CYTOMEGALOVIRUS AND EPSTEIN-BARR VIRUS IN LEUKAEMIAS AND LYMPHOMATA

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Andersen, P., Molgaard, J., Andersen, I. & Andersen, H. K. Smooth-muscle antibodies and antibodies to cytomegalovirus and Epstein-Barr virus in leukaemias and lymphomata. Acta path. microbiol. scand. Sect. C, 84 86–92 1976

By means of the indirect immunofluorescent method, smooth-muscle antibodies (SMA) were detected significantly more often in patients with malignant diseases (11.7 per cent) than in normal controls (3.3 per cent) ($0.02 > p > 0.01$). SMA occurred in 23.8 per cent of patients with Hodgkin's disease, in 13.3 per cent of patients with myeloproliferative diseases and in 4.7 per cent of patients with lymphoproliferative diseases. Other tissue antibodies were rare and they were found to occur at the same frequency in patients and controls. The occurrence of cytomegalovirus (CMV) antibodies did not differ significantly in patients and controls and, in both groups, the frequency of these antibodies increased with increasing age. The frequency of Epstein Barr-virus (EBV) antibodies increased also with increasing age both in patients and controls, but these antibodies were found more frequently in patients than in controls ($p = 0.03$). No relationship between the occurrence of SMA and viral antibodies was demonstrated. Thus, the development of SMA in patients with malignancies could not be shown to be due to CMV or EBV infection.

Key words: Antibodies; smooth-muscle; cytomegalovirus; Epstein-Barr virus; leukaemia.

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The incidence of antibodies reacting with certain tissue constituents has been demonstrated to be increased in patients with various malignant diseases (14, 16, 18, 19). Thus, the incidence of smooth muscle antibodies (SMA) has been found to be above normal range in patients with breast cancer, leukaemia and lymphoma (18, 16), but the reason why SMA occur in these diseases is not clear. However, SMA have also been found in cytomegalovirus (CMV) infection and in infectious mononucleosis (3, 4, 10) and it cannot be excluded that infection with CMV or Epstein Barr virus (EBV) might elicit the formation of SMA and other tissue antibodies in patients with neoplastic disease as infectious complications are frequent in these patients (13). With a view to examining this we have studied the relationship between tissue antibodies and CMV and EBV antibodies in patients with leukaemias and lymphomata.

TABLE 1 *Diagnoses Sex and Age Distribution and the Occurrence of CMV and EBV Antibodies in 94 Patients with Malignant Diseases and in 152 Controls*

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Sera from patients and controls had been stored at 20 C for up to [illegible] years. The diagnoses of the patients as well as the age and sex distribution of patients and controls are shown in Table 1. The patients can be grouped into three main categories: Hodgkin's disease, lymphoproliferative diseases and myeloproliferative diseases (13). The group of patients with lympho-reticulosarcoma comprised six cases of lymphosarcoma, one case of lymphoma giganto-folliculare, four cases of reticulosarcoma and two cases of lympho-reticulosarcomatosis. The majority of the patients were under treatment with cytotoxic drugs (cyclophosphamide, vincristine, cytosine arabinoside, daunomycin, melphalan, chlorambucil, and amethopterin) and steroid hormones. Twenty-eight patients had been treated with local irradiation within six months prior to the serological investigation. Sixty-five of the patients were only examined once, while 29 patients were examined on two or three occasions at intervals of from one to 28 weeks. All sera from one patient were studied in the same experiment.

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Viral Antibodies

Cytomegalovirus (CMV) antibodies were detected by a complement fixation test using a *microtitre system*. The antigen was produced in human lung-cell cultures with CMV laboratory strain Ad 169 (15). 4 units of antigen and 1½ units of complement were used for the test. Prior to investigation, the *sera were inactivated at 56° C* for 30 min and the lowest serum dilution tested was 1:4. The amount of non-haemolysed cells was estimated to range from 0 to 4+, and specimens showing 3+ or 4+ were considered positive. Positive sera were titrated in doubling dilutions starting at 1:4.

Epstein-Barr virus (EBV) antibodies were detected by IIF employing acetone-fixed EBV infected cells as antigen. Slides with antigen-containing cells were prepared from the P3HR-1 cell line (9). The cells were grown in Eagle's minimal essential medium containing 8 per cent *foetal calf serum*, 200 i.u. penicillin and 200 μg streptomycin per ml. Before the cells were harvested *with a view to producing slides*, they were starved for five days by changing the medium to Hank's balanced salt *solution containing 5* per cent foetal calf serum and antibiotics. After washing in phosphate-buffered saline (PBS), 5×10^4 *cells* were placed on rinsed slides and allowed to dry. After fixation in water-free acetone at room temperature for 10 min, the slides were stored in sealed boxes at –70° C until use. Sera were tested in a dilution of 1:10 in PBS. Antigen discs were covered with the serum dilution and incubated in a moist chamber at 37° C for 45 min. After washing in PBS, the slides were incubated with a *heterospecific FITC*-conjugated anti-human immunoglobulin (Wellcome Research Laboratories) for 30 *min. After washing in PBS, the preparations* were mounted in 50 per cent glycerol in PBS; a cover slip was applied and the specimens were examined under a Leitz fluorescence microscope (Orthoplan) with incident illumination and a HBO 200 mercury-vapour lamp. The excitation filter was a combination of BG38/KG1 and a KP490 interference filter. A K510 dichroic beam splitting mirror with a K515 barrier filter was employed, and a K510 was used as an additional barrier filter. The results of the test were expressed *either as positive* or negative.

RESULTS

Occurrence of CMV and EBV Antibodies

CMV antibodies were demonstrated in 55 per cent of the controls and in 53 per cent of the patients (Table 1). Sera *from one patient and from one control were anticomplementary and accordingly CMV antibodies* could not be determined. The frequency of CMV antibodies in the groups of patients ranged from 40 per cent to 68 per cent, and it appears from Table 2 that the frequency of CMV antibodies both in patients and controls increased with increasing age.

EBV antibodies were found in 72 per cent of the controls and in 81 per cent of the patients; the frequency of EBV antibodies ranged from 40 per cent to 100 per cent in the different groups of patients. The frequency of EBV antibodies increased with age from 0 to 20 years both in patients and controls (Table 2); in six out of seven age groups, however, EBV antibodies occurred

TABLE 2. *Number of Sera with CMV and EBV Antibodies Related to Age of 94 Patients with Malignant Diseases and 152 Controls*

		No. of subjects with antibody / No. of subjects tested						
		Age in years						
		0–9	10–19	20–29	30–39	40–49	50–59	> 60
No. of subjects with CMV antibodies (%)	Controls	1/6 (17%)	2/5 (40%)	22/47 (47%)	13/26 (50%)	15/26 (58%)	18/27 (72%)	12/15 (80%)
	Patients	3/10 (33%)	3/10 (30%)	6/12 (50%)	3/10 (33%)	9/16 (56%)	11/15 (73%)	12/21 (57%)
No. of subjects with EBV antibodies (%)	Controls	2/6 (33%)	3/5 (60%)	36/47 (77%)	19/26 (73%)	18/26 (69%)	19/27 (70%)	12/15 (80%)
	Patients	*5/10* (50%)	*6/10* (60%)	*10/12* (83%)	*9/10* (90%)	*14/16* (88%)	*13/15* (87%)	20/21 (95%)

TABLE 3. *Tissue Antibodies in Patients with Malignant Diseases and in Controls*

Diagnosis	No. of subjects	Number of patients with SMA *	GA	ANA	PA	One antibody or more
Hodgkin disease	21	5 (23.8%)	1	0	1	5
Lymphoproliferative diseases	43	2* (4.7%)	1	2	2	5
Myeloproliferative diseases	30	4‡ (13.3%)	0	0	0	4
All patients	94	11 (11.7%)	2 (2.1%)	2 (2.1%)	3 (3.2%)	14 (14.9%)
Controls	152	5 (3.3%)	1 (0.7%)	5 (3.3%)	4 (2.6%)	13 (8.5%)

SMA, smooth-muscle antibody GA, glomerular antibody ANA, antinuclear antibody PA, parietal-cell antibody

* SMA were present in one pt. with acute lymphoblastic leukaemia and in one with myelomatosis.

‡ All SMA-positive patients had acute myeloblastic leukaemia.

significantly more frequently in patients than in controls (sign test, $p = 0.03$)

Occurrence of SMA and other Tissue Antibodies

The incidence of tissue antibodies in patients and controls is shown in Table 3. If more than one serum sample from a patient had been investigated, only the results of the first sample are included in Table 3. SMA were found in 11 (11.7 per cent) of 94 patients with malignant diseases and only in 5 (3.3 per cent) of 152 controls (chi-square test, $0.02 > p > 0.01$). It is also seen that the incidence of SMA was higher in patients with Hodgkin's disease (23.8 per cent) and in patients with myeloproliferative diseases (13.3 per cent) than in patients with lymphoproliferative diseases (4.7 per cent) and in controls (3.3 per cent). However the differences in the three patient categories were not significant (chi-square test $0.10 > p > 0.05$). This could be due to the low number of patients tested. SMA were found in one out of 28 patients (3.5 per cent) who had received irradiation and in 10 out of 66 patients (15.2 per cent) who had not been irradiated. Still, this difference was not significant. The SMA were of the IgG class in all patients, and the titres ranged between 10 and 40. In the controls, SMA were of the IgG class in four cases, while one serum contained IgG- as well as IgM-SMA. The titres were 10 and 20.

IgG-GA were found in two patients whose

TABLE 4. *The Occurrence of Smooth-muscle Antibodies in Sera with and without CMV and EBV Antibodies*

	No. of subjects with SMA / No. of subjects tested (SMA-pos. %): CMV antibody negative	CMV antibody positive	EBV antibody negative	EBV antibody positive
Patients with malignant disease	4/43 (9.3%)	7/50 (14.0%)	1/18 (5.6%)	10/76 (13.2%)
Controls	3/69 (4.3%)	2/82 (2.4%)	2/43 (4.7%)	3/109 (2.7%)

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	Patients	3/10 (33%)	3/10 (30%)	6/12 (50%)	3/10 (33%)	9/16 (56%)	11/15 (73%)	12/21 (57%)
No. of subjects with EBV antibodies (%)	Controls	2/6 (33%)	3/5 (60%)	36/47 (77%)	19/26 (73%)	18/26 (69%)	19/27 (70%)	12/15 (80%)
	Patients	5/10 (50%)	6/10 (60%)	10/12 (83%)	9/10 (90%)	14/16 (88%)	13/15 (87%)	20/21 (95%)

serum at a higher rate than Paul-Bunnell heterophil antibodies and CMV antibodies (3, 4). Only SMA of the IgG class were found in patients with malignant diseases this might explain why a correlation between the occurrence of SMA and CMV antibodies could not be established in the present study. However it cannot be precluded that infectious agents other than CMV and EBV were responsible for the formation of SMA (6). ANA have also been demonstrated in the course of infectious mononucleosis and CMV infection (4 12). The fact that no correlation between ANA and viral antibodies was found might also support the view that factors other than infection with CMV and EBV are responsible for an increased incidence of SMA in haematological malignancies. SMA are primarily found in liver disease (17) and thus, a hepatic involvement could possibly explain the occurrence of SMA in some patients.

Whitehouse & Holborow (19) suggested that the presence of SMA in various malignant diseases was related to changes in the malignant cell membrane. Actomyosin-like proteins, against which SMA are directed, occur in many cell types (7), alterations in these cells might elicit the production of antibodies that react with smooth-muscle components.

In some cases, local irradiation has been followed by the development of auto-antibodies (11). However this could not explain the increased incidence of SMA in our patients with haematological malignancies as SMA occurred less frequently in patients who had received irradiation (5).

Most patients were under treatment with cytotoxic drugs many of which can reduce antibody production (5). It is to be expected that the production both of viral antibodies and SMA would be affected by these drugs. Thus, the lack of correlation between viral antibodies and SMA can hardly be explained by the treatment.

Leukaemia and lymphomata are associated with immunological abnormalities and the ability to form antibodies seems to be impaired to a greater extent in lymphoproliferative diseases than in Hodgkin's disease and myeloproliferative diseases (8 13). The occurrence of CMV and EBV antibodies was not significantly reduced in our patients with lymphoproliferative diseases, but an impaired humoral immunity might explain why SMA were rare in these patients. It is not clear however why SMA were found in the other malignant diseases and it was not possible to relate the occurrence of this antibody to CMV or EBV infection.

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REFERENCES

1. *Andersen P & Beutner E. H.* Defined immunofluorescence studies of cytoplasmic antibodies. Int. Arch. Allergy 43 780–790 1972.
2. *Andersen P.* Indirect immunofluorescence studies of smooth muscle antibodies. Acta path. microbiol. scand. Sect B 82 577–584 1974.
3. *Andersen P.* Smooth-muscle antibodies in sera with Paul-Bunnell heterophil antibodies. Acta path. microbiol. scand. Sect. C, 83 19–27 1975.
4. *Andersen P & Andersen H K.* Smooth-muscle antibodies and other tissue antibodies in cytomegalovirus infection. Clin. exp. Immunol. 22 22–29 1975.
5. *Berenbaum M C.* A screen for agents inhibiting the immune response and the growth of tumours. Nature 196 384 385 1962.
6. *Farrow L J., Holborow E. J., Johnson G D., Lamb S G. Stewart J S., Taylor P E. & Zuckerman A J.:* Autoantibodies and the hepatitis-associated antigen in acute infectious hepatitis. Brit. Med. J 2 693–695 1970.
7. *Farrow L J Holborow E J & Brighton, W D.* Reaction of human smooth muscle antibody with liver cells. Nature new Biol. 232 186–187 1971.
8. *Heath R B Fairley G H & Malpas J S.* Production of antibodies against viruses in leukemia and related diseases. Brit. J Haemat. 10 365–370 1964.
9. *Hinuma Y Konn M Yamaguchi J., Wudarski D J Blakeslee J R J & Grace J T., J.* Immunofluorescence and herpes-type virus particles in the P3HR 1 Burkitt lymphoma cell line. J Virol. 1 1045–1051 1967.

TABLE 5 *CMV Antibody Titres and SMA Titres in four Patients with Malignant Disease*

Antibody	First sample	Weeks after first sample				Diagnosis
		2–4	5–10	11–20	21–30	
CMV Ab	16			8	256	
SMA	< 10			< 10	< 10	Acute lymphoblastic leukaemia
CMV Ab	4	4		128		
SMA	< 10	< 10		< 10		Acute lymphoblastic leukaemia
CMV Ab	4	*				
IgG-SMA	< 10	20				Acute lymphoblastic leukaemia
CMV Ab	< 4	< 4				
IgG-SMA	< 10	40				Acute myeloblastic leukaemia
IgA-SMA	< 10	80				

* Serum was anticomplementary and CMV antibodies were not determined

serum also contained SMA, and in one control subject in whom SMA were absent ANA and PA were rare and were demonstrated to occur at the same frequency in patients and controls. The ANA titres ranged between 20 and 160 the antibodies were of the IgG and IgA class in the patients and of the IgG and IgM class in the controls. All PA were of the IgG class, the titres ranging between 20 and 80 MTA were not found either in patients or controls.

Relationship between SMA and Viral Antibodies

In patients with malignant disease SMA were found more often in sera containing CMV antibodies and EBV antibodies than in sera where these antibodies were absent as opposed to the controls in whom SMA occurred most frequently in sera not containing CMV and EBV antibodies (Table 4) still these differences were not significant. It was not possible either to establish any relationship between the occurrence of SMA and the height of the CMV antibody titres.

On two or three occasions at intervals of from one to 28 weeks, 29 patients were examined with a view to a presence of tissue antibodies and viral antibodies. The occurrence of EBV antibodies was not found to change in any of these patients. Any significant change (fourfold) in CMV antibody titres or SMA titres was not observed in 22 patients and SMA were only demonstrated in two of the latter In three patients, the SMA titre fell from 10 to below 10 within 2-16 weeks, while the CMV-antibody titre remained unchanged. A significant change in antibody titres was demonstrated in four patients. The serological findings in these patients are shown in Table 5 In two patients, a fourfold increase or above of the CMV antibody titre was observed, but tissue antibodies could not be demonstrated in any of the serum samples. In the third patient, the IgG-SMA titre increased from below 10 up to 20 within three weeks. The CMV-antibody titre was 4 in the first sample but CMV antibodies could not be determined three weeks later because the serum had become anticomplementary probably because of the development of antigen-antibody complexes. In the last patient, two serum samples obtained three weeks apart were negative for CMV antibodies. The first sample did not contain SMA while IgG as well as IgA-SMA in titres of 40 and 80 respectively were present in the second sample

DISCUSSION

SMA in infectious mononucleosis and CMV infection are most often of the IgM class they occur transiently and disappear from the

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ACUTE DISSEMINATED PHYCOMYCOSIS IN A PATIENT WITH IMPAIRED NEUTROPHIL GRANULOCYTE FUNCTION

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A 13-year-old girl with no previously known predisposing disease developed phycomycosis involving the left lung, pleura and shoulder the left side of the neck, the left thigh, the kidneys and the brain. Prolonged amphotericin B therapy resulted in clinical improvement, but the disease was wide-spread when the patient died 5 months after debut of symptoms from a subarachnoid haemorrhage due to fungal destruction of the basilar rtery During hospitalization, a marked reduction in the bactericidal activity of circulating neutrophil granulocytes was repeatedly demonstrated and the endotoxin stimulated nitroblue tetrazolium test was negati.. Together with the demonstration of granuloma formation and the accumulation of lipid-laden histiocytes in the spleen, lymph nodes, bone marrow and the thymus, these findings indicate that the patient had les severe form of chronic granulomatous disease.

Key ords Phycomycosis; neutrophil granulocytes impaired function.

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The term phycomycosis is applied to fungal infections caused by *Mucor* and related fungi presenting identical morphology on histological examination (11 21) Phycomycosis has most frequently been found in patients with diabetic acidosis, and infections of the respiratory tract and the central nervous system are most common (21) Recently the incidence has been reported to be increased in patients with leukaemia and malignant lymphomas (14) Two patients without any known predisposing disease have also been described (8, 21)

In the present study acute disseminated phycomycosis was found to be manifest in a previously healthy girl in whom also the phagocyte function was disordered, characterized by impaired intracellular killing of microorganisms by neutrophil granulocytes.

CASE REPORT

The patient, a 13-year-old girl, was no. 5 of 8 siblings. Her parents and siblings were all healthy

10 *Holborow E J Hemsted E. H & Mead S I* Smooth muscle autoantibodies in infectious mononucleosis. Brit Med J *3* 323-325 1973
11 *Jonsson J Einhorn Nina Fagraeus Astrid & Einhorn J* Organ antibodies after local irradiation. Radiology *90* 536-539 1968
12 *Kaplan M E & Tan E M* Antinuclear antibodies in infectious mononucleosis. Lancet *i* 561-563 1968.
13 *Miller D G* Patterns of immunological deficiency in lymphomas and leukemias. Ann. Intern Med. *57* 703-716 1962
14 *Moore M & Hughes L A* Circulating antibodies in human connective tissue malignancy Br J Cancer *28* Suppl. I 175-184 1973
15 *Rowe W P Hartley J W Waterman S Turner H C & Huebner R. J* Cytopathogenic agent resembling salivary gland virus recovered from tissue cultures of human adenoids. Proc. Soc. exp. Biol. (N.Y.) *92* 418-424 1956.
16 *Tannenberg A E G Muller H K, Cauchi, M N & Nairn R C* Incidence of autoantibodies in cancer patients. Clin. exp. Immunol *15* 153-156 1973
17 *Fischer T L* Australia antigen and autoantibodies in chronic hepatitis. Brit. Med. J *2* 695-698 1970
18. *Wasserman J., Glas Ulla & Blomgren H* Autoantibodies in patients with carcinoma of the breast. Clin. exp Immunol. *19* 417-422, 1975
19 *Whitehouse J M A & Holborow E J* Smooth muscle antibody in malignant disease. Brit. Med. J *4* 511-513 1971

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Fig 1 Chest X-ray showing infiltrate of the lateral part of the left upper lung field

She had previously and intermittently been suffering from localized dermatitis thought to be atopic eczema. However cutaneous symptoms had not been in evidence for the last 2–3 years prior to the present illness Several years ago she had morbilli and pertussis.

Onset of the present illness in December 1972 was in the form of dermatitis of the scalp Therapy with steroid ointment was unsuccessful Two weeks later transient urticaria pain in the left shoulder intermittent fever and increasing left cervical and axillar lymphnode enlargement developed Chest X ray showed a density in the upper lateral area of the left lung Despite therapy with various antibiotics, the condition deteriorated and following aspiration of 450 ml blood-stained fluid from the left pleural cavity the patient was admitted to our hospital on February 17 1973

On admission, the body temperature was 39 6 C a Horner's syndrome was present on the left side the density visualized by chest X ray had increased (Fig 1) and a mass, the size of an orange, was felt anterolaterally in the middle of the left thigh. Pertinent laboratory data included Hgb 9 2 g 100 ml ESR 92 mm/h, platelet count 765000 mm³ WBC 46800/mm³ with 78 per cent segmented neutrophil granulocytes, 16 per cent band forms 5 per cent lymphocytes and 1 per cent eosinophils During the first week, eosinophil granulocyte counts increased to 5190/mm³ Bone marrow aspirates were normal except for eosinophilia and a slight shift to the left of the myeloid cells. Serum electrophoresis revealed hypoalbuminaemia and slight elevation of gammaglobulins. Immunoelectrophoresis showed a normal pattern except for elevation of IgA (3 06 µg/ml) Renal function tests were normal The PPD-test was negative and the same applies to intradermal tests with candida, streptococcus, mumps and aspergillus antigens However the DNCB (dinitrochlorobenzene)-test for delayed hypersensitivity was positive. C3 and C4 levels were normal. Multiple blood cultures and pleural fluid cultures gave no growth. Cold agglutinin titres varied between 32 and 64 but mycoplasma antibodies were not present. The following tests were negative I E-cells, ANF anti-DNA, Waaler Rose, Latex Ra and toxoplasma antibodies. Incubation of the patient's granulocytes in the presence of pooled human serum to serve as opsonin and *Staphylococcus aureus* to serve as test bacterium (technique see 18) demonstrated only a slight reduction in total number of viable bacteria as with compared findings in a test using granulocytes from a normal control besides, large numbers of viable bacteria were located intracellularly indicating normal phagocytosis but significantly reduced intracellular killing of bacteria (Fig 2) The NBT (nitroblue tetrazolium) test (technique see 12) by which the reduction within the leucocytes of NBT-dye to formazan, is measured, was negative also in the presence of endotoxin stimulation. The tests of the granulocyte

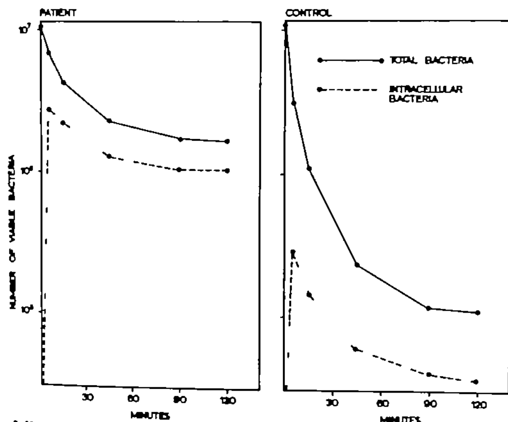


Fig. 2 Number of viable bacteria during incubation of granulocytes from the patient and a normal control. Mean of three experiments, one on each of three consecutive days. The technique has previously been described in detail (18). Briefly 0.1 ml bacteria suspension (10–14 × 10^7 *Staphylococcus aureus* "Oxford"—Heatley strain) was incubated at 37° C with 0.5 ml leucocyte suspension containing 5 × 10^6 polymorphonuclear granulocytes and 0.4 ml pooled human serum diluted 1:3 in Hank' balanced salt solution. The total number of viable bacteria and the number of viable intracellular bacteria were periodically determined.

function were later repeated on five different occasions, each time giving the same results as those obtained on admission to hospital. Preliminary examination of biopsy specimens obtained from the cervical lymph nodes and the infiltrate of the left thigh showed necrosis, inflammatory changes with abundant eosinophils and areas with vasculitis and necrosis of vessel walls. The inflammatory reaction was partly diffuse, partly granulomatous with many giant cells. These findings suggested an acute allergic inflammation. Accordingly high doses of corticosteroids and cyclophosphamide 200 mg daily were administered.

Despite temporary improvement, the density of the left lung field persisted and a swelling of the left shoulder developed. Thoracic surgical exploration revealed inflammatory changes with areas of necrosis extending from the subscapular region through the thoracic wall to the pleura and adjacent part of the left upper lobe of the lung. Further examination of the primary and supplementary biopsies revealed the presence of broad, nonseptate irregular branching mycelial threads in the tissues, vessel walls, within the giant cells and in the necrotic areas. The threads stained darkly with haematoxylin and eosin (HE) but showed rather weak staining with periodic-acid-Schiff (PAS) and Gomori' methenamine-silver (GMS) technique (Figs. 3 a, b, c). These findings prompted the diagnosis of acute disseminated phycomycosis. The material was later reviewed at the Infectious Disease Branch at the Armed Forces Institute of Pathology in Washington, USA, and the morphological diagnosis was confirmed.

Therapy with amphotericin B in doses gradually increasing from 5000 to 40000 IU i.v. daily was instituted, later every second day for nine weeks altogether. After therapy for five weeks, improve-



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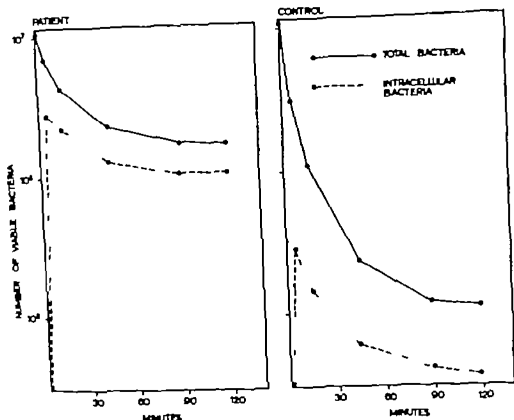
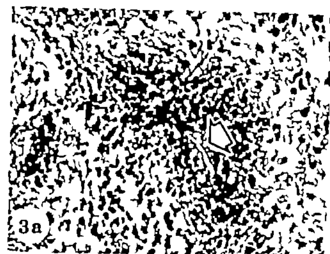


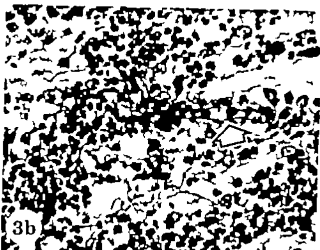
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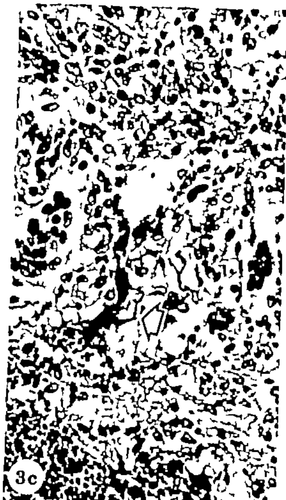


Fig 3 Biopsy from the left thigh. *a*. Central hyphae of a Phycomycete (arrow) with surrounding eosinophilic amorphous material and granulocytic infiltration with many eosinophils. *b* Necrotic muscle fibres heavily infiltrated with eosinophils. Broad darkly stained, non- septate hyphae (arrow) *c* Mycelial threads (arrow) inside foreign body giant cell (HE × 280)

ment became apparent the body temperature spikes decreased, the inflammatory changes over the left shoulder diminished and the patient was able to take food orally However a bronchial fistula and empyema of the left pleura developed the latter requiring surgical drainage and antibacterial therapy The infiltrate of the left thigh progressed to necrosis and the middle and lateral parts of the quadriceps muscle were removed. Later as subcutaneous emphysema, fistulation to the left axillar and supraclavicular regions and impaired renal function became evident cotrimazol was substituted for amphotericin B However only one week later the patient suddenly died of subarachnoid haemorrhage

Autopsy revealed a wide-spread purulent inflammation with severe necrosis. The latter was mainly localized to the left side of the thorax, left pleural cavity the neck where the processi spinosi were necrotic, the left thigh, lymph nodes and the kidney Furthermore examination of the brain showed that an inflammatory infiltrate in the pons region had caused necrosis of the basilar artery with massive subarachnoid haemorrhage. Imprints from the inflammatory tissue in pons (Fig 4) and histological sections from necrotic areas all showed fungal elements. The surface of the kidneys showed multiple small yellow nodules. Microscopy of these revealed granulomatous lesions with and without central necrosis (Fig 5) Some of these granulomas showed fungal elements. Sections from the spleen, lymph nodes, bone marrow and the thymus showed abundance of histiocytes containing pigment with staining characteristics of lipochrome (Fig 6 a, b)

DISCUSSION

Phycomycosis seems to be an uncommon infection and to the best of our knowledge, this is the first Scandinavian case described. Cul



Fig. 4 Imprint from pons lesion with broad, nonseptate hyphae with characteristic branching (HE × 700)



Fig. 5 Kidney tissue with histiocytic granulomatous lesion (HE × 175)

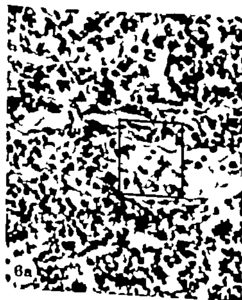




Fig. 6 a. Spleen tissue with large histiocytes containing lipochrome pigment (HE × 280) b Larger magnification (× 1120) of inset in a.

ture of the fungi is difficult and as in the present case it is usually unsuccessful (14 16) The diagnosis is usually based upon the demonstration of typical mycelial threads in biopsy specimens. The broad irregular, non-septate, mycelial threads, often with right angle branching as demonstrated in the microscopic sections from our patient, are regarded as pathognomonic (11 14 16)

The infection may be disseminated as in our patient, or it may be localized e g to the sinuses, the central nervous system or the lungs. In most of the described cases the infection occurred in patients in whom the immune defence mechanisms were impaired—particularly in diabetics with keto-acidosis (1 5 11) and in patients with malignant lymphomas or leukaemia (14) Our patient had no previously known predisposing disease. However since infancy she had suffered from dermatitis and during her stay in our hospital a marked and permanent reduction in the bactericidal activity of the granulocytes was demonstrated and the endotoxin stimulated NBT test was negative. Together with the demonstration of granuloma formation and the accumulation of lipid laden histiocytes in various internal organs, these findings indicate that the patient had a less severe form of chronic granulomatous disease (CGD) (2 9 15 17) Other conditions involving permanently impaired phagocyte function such as myeloperoxydase (13 17) and glucose-6-phosphate dehydrogenase (3 17) deficiencies could be excluded on the basis of clinical and biochemical investigations. In early myelomonocytic or myeloblastic leukaemia reduced granulocyte function may be demonstrated before it is possible to have the diagnosis established on the basis of clinical and haematological findings (19) However in our patient there was no evidence of leukaemia either during her stay in hospital for more than three months or at autopsy Furthermore, the granulocyte dysfunction and possible leukaemoid reaction seen in severe infection could also be excluded since this is a transient phenomenon which is reversed following adequate antimicrobial therapy and normalization of white blood cell morphology (20) Finally transient granulocyte dysfunction has been demonstrated in leukaemia patients exposed to craniospinal irradiation (4) and also in a patient with cryoglobulinaemia (10) Our patient had not received X ray therapy and cryoglobulinaemia was not in evidence. Accordingly in the absence of evidence of abnormal immune functions for 13 years our patient most likely had CGD

Therapy of disseminated phycomycosis is difficult Amphotericin B seems to be the best therapeutic agent (5 6) Cotrimazol has also good *in vitro* effect (7 22) but the *in vivo* effect is more doubtful (7) Surgical drainage and removal of necrotic material are often necessary In our patient, definite clinical improvement could be demonstrated during amphotericin B therapy However despite therapy for nine weeks, the disease was widespread when the patient died of subarachnoid haemorrhage due to dissemination to the central nervous system.

REFERENCES

1 *Abramson E Wilson D & Arky R A* Rhinocerebral phycomycosis in association with diabetic ketoacidosis. Ann Intern. Med. *66* 735 742 1967

2. *Azimi P Bodenbender J G., Hintz R L & Kontras S B* Chronic granulomatous disease in three sisters Lancet *I* 208 209 1968.

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6 *Bennett J E* Chemotherapy of systemic mycoses. New Engl J Med *290* 30 32 1974

7 *Bennett J E.* Chemotherapy of systemic mycoses. New Engl. J Med. *290* 320–323 1974
8 *Blankenberg, H W & Verhoff D.*. Mucormycosis of the lung Amer Rev T berc. *79* 357 361 1939
9 *Douglas, S D Davis, W C & F denberg H H* Granulocytopathies Pleomorphism of neutrophil dysfunction. Amer J Med. *46* 901 909 1969
10. *Douglas, S D Lahav M & F denberg H H* A reversible neutrophil bactericidal defect associated with mixed cryoglobulin. Amer J Med. *49* 274 280 1970.
11 *Emmons C W Binford C H & Utz J P.*: Medical mycology Lea & Febiger Philadelphia 1970.
12. *Hellum K. B. & Solberg C O*: Influence of anticoagulants on the nitroblue tetrazolium test Scand. J Infect. Dis. *5* 67–70, 1973
13. *Klebanoff S J & Hamon C B.* Role of myeloperoxidase-mediated antimicrobial systems in intact leukocytes. J Reticuloendothel. Soc. *12* 170–196, 1972.
14. *Meyer R. D R sen P & Arm ong, D* Phycomycosis complicating leukemia and lymphoma Ann. Intern Med. *77* 871–879, 1972.
15. *Nathan D G.* NBT reduction by human phagocytes. New Engl. J Med. *290* 280–281 1974
16. *Osber A G & Remington J S.*: Phycomycosis. In Hoeprich, P D. (Ed.): Infectious diseases. Harper & Row New York 1972, p. 971 973
17 *Quie P G* Disorders of phagocyte function. Year Book Medical Publishers, Chicago 1972.
18. *Solberg C O*: Enhanced susceptibility to infection. A new method for the evaluation of neutrophil granulocyte f nctions. Acta path. microbiol. scand. Sect. B *80* 10–18, 1972.
19 *Solberg C O Sch einer A., Hell m A. B. & Ham e E.*. Neutrophil granulocyte function in the early diagnosis of acute myelomonocytic and myeloblastic leukaemia. Acta med. scand. *197* 147 151 1975
20 *Solberg C O & H ll m A B.*. Neutrophil granulocyte function in bacterial infections. Lancet *II* 727–730, 1972
21 *St atton, B. R Zimmerman L. E. & Gass, J D M* Phycomycosis. A clinicopathologic study of fifty-one cases. Lab. Invest. *11* 963–985 1962.
22. *H gman T* Therapie der Systemmykosen. Infektion *1* 88–91 1973.

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PREPARATION OF PMN MONOLAYERS	PMN (8×10^6/ml) suspended in KRG on tissue culture tubes 60 min at 37° C.
↓	
INGESTION	Incubation with ^{32}P-labelled *E. coli* (10^9/ml) in KRG ± serum, 15 min at 37° C
↓	
WASHING	of monolayers with KRG at 37° C (×4)
↓	
(DIGESTION) + ELIMINATION	Incubation in KRG ± serum for 0–180 min in the postingestive period at 37° C. Determination of extracellular and intracellular label. Determination of cell protein.

Fig. 1. Outline of method for assessing elimination of bacterial label from PMN-monolayers in the postingestive period.

before fresh KRG medium with or without 10 per cent rat serum was added and further incubated at 37° C for 180 min.

Determination of Extracellular Label in the Postingestive Period

Aliquots of 0.1 ml of the medium were withdrawn from the tissue culture tubes at different intervals for determinations of label (16) expulsed from phagocytes into the medium. At the end of the test period, radioactivity was measured both on centrifuged (6000 × g/10 min/0° C) and uncentrifuged medium.

Determination of Intracellular Label

was performed as previously described (16).

Examination of Monolayer by Light Microscopy

At the end of the ingestion period, the tissue culture tubes were washed as described, and 2.5 ml of a solution of 0.25 per cent safranin dye in 10 per cent ethanol was added. The dye was removed after 1 min and the tubes were gently rinsed in water. To facilitate microscopic examination of the "floor section" of the tubes (Fig. 3), the tubes were cut into 2 pieces.

Quantitation of Monolayers

was done by determination of the protein of the glass-adherent cells (16).

Calculations

1. *Elimination* (E) of label from washed PMN in the postingestive period was expressed as percentage of the total uptake of radioactivity per tube. At a certain time t, E = extracellular radioactivity/(intracellular + extracellular radioactivity) × 100.

2. *Ratio ingested bacteria/phagocyte.* The number of intracellular bacteria was calculated by comparing the intracellular radioactivity with the amount of radioactivity incorporated in a known number of bacteria. The final number of PMN per tube was calculated by assay of their cell protein compared with the quantity of protein of a known number of PMN washed and suspended in KRG.

Statistical Analysis

was carried out by means of the two-samples rank tests of Wilcoxon-White (10).

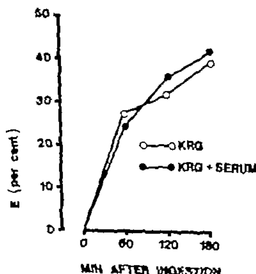


Fig. 2. Elimination (E) of ^{32}P from PMN in percentage of total uptake of ^{32}P-labelled *E. coli* in the presence ●——● and in the absence ○——○ of 10 per cent serum during ingestion. A standard period of incubation, 15 min at 37° C, with 10^9 of bacteria per ml was used. E was measured in the postingestive period (0–180 min). KRG was used as postingestive medium. Results of 2 experiments.

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ELIMINATION OF INGESTED ^{32}P-LABELLED *E COLI* FROM RAT POLYMORPHONUCLEAR NEUTROPHILS (PMN)

Evaluation of a Method

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Midtvedt T & Baardsen, A Elimination of ingested ^{32}P labelled *E coli* from rat polymorphonuclear neutrophils (PMN) Evaluation of a method Acta path. microbiol. scand Sect. C, 100-104 1976

A method to be used for *in vitro* assay of the elimination of ingested ^{32}P labelled *E. coli* from rat PMN monolayers has been evaluated The rate of expulsion of bacterial label from phagocytes into the extracellular medium was found to range between 40 and 50 per cent of the total uptake 180 min after termination of ingestion. Serum enhanced the cellular uptake of bacteria but did not affect the rate of elimination. Disintegration of the phagocytes was not found to be a problem.

Key words Neutrophils phagocytosis *E. coli*.

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Elimination of bacterial breakdown products from phagocytes is the phase of phagocytosis least throughly studied (7 8 13)

Degradation of isotopically labelled bacteria by PMN has been studied by *Cohn* (3 4) A method by which the elimination of bacterial breakdown products from living PMN might be measured however was not developed

The aim of the present study has been to evaluate a method for determination of bacterial label eliminated from rat peritoneal PMN after ingestion of ^{32}P labelled *E. coli*

MATERIALS AND METHODS

The experimental model is outlined in Fig 1

Preparation of Rat Peritoneal PMN Monolayers

in tissue culture tubes was done as previously described (16)

Ingestion

The uptake of P-labelled *E coli* was measured according to a method previously described (16) A standard period of incubation of 15 min at 37 C with 10^9 per ml of *E coli* was used. Krebs-Ringer phosphate buffer with 10 mM glucose (KRG) was used as basic medium, and serum from conventional rats (1) was added as stated in legends to Fig 1-4

Postingestive Incubation

At the end of ingestion the monolayers were quickly washed 4 times in 2.5 ml KRG at 37 C

PREPARATION OF PMN MONOLAYERS	PMN (8×10^6/ml) suspended in KRG on tissue culture tubes, 60 min at 37 C.
↓	
INGESTION	Incubation with ^{32}P-labelled *E. coli* (10^9/ml) in KRG ± serum, 15 min at 37 C
↓	
WASHING	of monolayers with KRG at 37 C (×4)
↓	
(DIGESTION) + ELIMINATION	Incubation in KRG ± serum for 0-180 min in the postingestive period at 37 C. Determination of extracellular and intracell lar label Determination of cell protein.

Fig 1 Outline of method for assessing elimination of bacterial label from PMN-monolayers in the post ingesti period

before fresh KRG medium with or without 10 per cent rat serum was added and further incubated at 37° C for 180 min.

Determination of Extracellular Label in the P stingestiv Period

Aliquots of 0.1 ml of the medium were withdrawn from the tissue culture tubes at different intervals for determinations of label (16) expulsed from phagocytes into the medium. At the end of the test period, radioactivity was measured both on centrifuged (6000 × g/10 min/0° C) and uncentrifuged medium

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Quantitation f Monolayer

as done by determination of the protein of the glass-adherent cells (16)

Calculations

1 *Elimination* (E) of label from washed PMN in the postingesti period was expressed as per centage of the total uptake of radioactivity per tube. At certain time t, E = extracellular radioactivity$_t$/intracellular + extracellular radioactivity × 100

2 *Ratio ingested bacteria/phagocyte* The number of intracellular bacteria was calculated by comparing the intracellular radioactivity with the amount of radioactivity incorporated in a known number of bacteria. The final number of PMN per tube was calculated by assay of their cell protein compared with the quantity of protein of a known number of PMN washed and suspended in KRG.

Statistical Analys

was carried out by means of the two-samples rank tests of Wilcoxon-White (10)

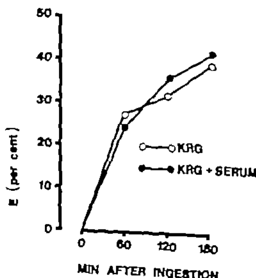


Fig Elimination (E) of ^{32}P from PMN in per centage of total uptake of ^{32}P-labelled *E. coli* in the presence ●——● and in the absence ○——○ of 10 per cent serum during ingestion. A standard period of incubation, 15 min at 37 C, with 10^9 of bacteria per ml was used. E was measured in the postingesti period (0-180 min) KRG was used as postingestive medium. Results of 2 experiments.

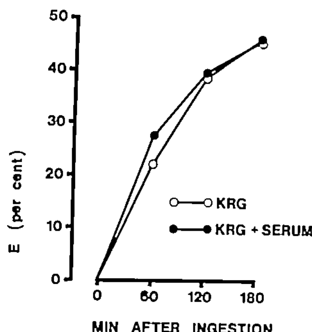


Fig 3 See legend to Fig 2 The figure illustrates the effect of presence or absence of 10 per cent serum in the postingestive period, after uptake under standard conditions in the presence of serum Results of 2 experiments.

RESULTS

Uptake of Bacteria by PMN

The number of ingested bacteria per phagocyte was 37 ± 7 0 (1 standard deviation) in the presence of serum, and 7 6 ± 3 1 (1 standard deviation) in the absence of serum.

Elimination of Bacterial Label from PMN

E was found to approximate 40 per cent 180 min after termination of uptake of bacteria (Fig 2) The presence of serum during ingestion (Fig 2) or during the postingestive period (Fig 3) did not influence E significantly ($p > 0\ 10$)

The amount of extracellular radioactivity (eliminated from PMN) in the postingestive medium at a certain time was of the same magnitude whether or not the medium was centrifuged.

Quantity of Glass-adherent PMN Related to the Length of the Postingestive Period of Time

The quantity of glass-adherent PMN expressed as µg protein per tube, did not change significantly within the test period of 180 min (Fig 4) ($p > 0\ 10$) no significant loss of cells was observed during the test period.

Microscopic Examination

of glass-adherent PMN (Fig 5) after uptake of bacteria revealed that the cells were arranged in monolayers without aggregation of individual cells. Furthermore, all PMN contained a high number of bacteria. Bacteria located extracellularly were not observed with certainty

DISCUSSION

The described method to be used for the assay of E should be considered as an extension of a previously described method by which PMN ingestion of *E coli* may be de-

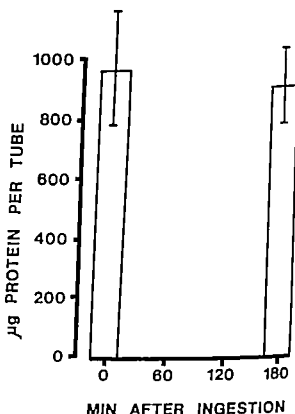


Fig 4 Protein content of cells (monolayer of PMN) per tube before and after a postingestive period of 180 min. Uptake under standard conditions in the presence of serum. KRG used as medium in the postingestive period Results of 2 experiments. I = ± 1 standard deviation.





Fig. 5 Microphotographs of PMN monolayer after uptake of *E. coli* a) × 250 b) × 1000.

termined (16). The use of glass-adherent PMN made it possible to separate postingestive events from the ingestion itself. It could be demonstrated that the phagocytes were *actually* arranged in monolayers. Thus, erroneous interpretation caused by aggregation of cells was avoided (14, 15). Furthermore, by washing of monolayers, error due to extracellular bacteria (12) was counteracted. The present in vitro system operates with a high ratio of ingested bacteria per phagocyte (strong stimulation) in contrast to other experimental methods (6, 12).

Measurement of E gave consistent results. Approximately 40 per cent of the total uptake of bacterial label was found in the extracellular medium 180 min after the uptake. The presence of serum during or after the ingestion did not appear to affect E, thus supporting the hypothesis that ingestion is the rate-limiting step in the intracellular degradation of *E. coli* (5). Apart from enhancing *ingestion, serum opsonins are apparently* not necessary for the elimination of labelled constituents of *E. coli* ingested by PMN in contrast to findings in macrophages (9, 11).

Re-incorporation of eliminated label into PMN might influence E. In a previous study it has been *found*, however, that re-incorporation is of minor importance in the present system (16).

Disintegration of phagocytes may be a consequence of phagocytosis (13) and might therefore interfere with the determination of E. This raises the question whether the extracellular radioactivity measured in the post

ingestive medium represents labelled bacterial constituents eliminated from intact PMN only or partly came from damaged cells. In the present test system, the latter alternative appears to be unlikely according to the unchanged quantity of cell protein at the end of the test period.

The intracellular killing of *E. coli* has been shown to occur much more rapidly than the morphological degradation of bacterial protoplasm (2, 5) and to be accompanied by a corresponding rapid increase in the permeability of the bacterial envelope (2, 5). Furthermore, loss of isotope from ^{32}P labelled *E. coli* correlates with loss of their viability (16). Therefore, it seems reasonable to assume that the label in the postingestive medium originates from bacteria which are killed during their stay within the phagocytes.

REFERENCES

1. *Baardsen A., Trippestad A. & Midtvedt T.*: *In vitro* influence of glucocorticosteroids on opsonic and bactericidal activities against *Escherichia coli* in rat serum. Acta path. microbiol. scand. Sect. B, *82* 696–700 1974.
2. *Beckerdite S., Mooney C., Weiss J., Franson R. & Elsbach P.*: Early and discrete changes in permeability of *Escherichia coli* and certain other gram negative bacteria during killing by granulocytes. J. Exp. Med. *140* 396–409 1974.
3. *Cohn Z. A.*: The fate of bacteria within phagocytic cells. I. The degradation of isotopically labeled bacteria by polymorphonuclear leucocytes and macrophages. J. Exp. Med. *117* 27–42, 1963.
4. *Cohn Z. A.*: The fate of bacteria within phagocytic cells. II. The modification of intracellular degradation. J. Exp. Med. *117* 43–53 1963.
5. *Elsbach P.*: On the interaction between phagocytes and microorganisms. N. Engl. J. Med. *298* 846–852, 1973.
6. *van Furth R. & van Zwet T. L.*: *In vitro* determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes. In: Weir D. M. (Ed.): Handbook of Experimental Immunology. sec. Ed. Vol. 2. Blackwell Sc. Publ. Oxford/London/Edinburgh, 1973 p. 36.1–36.23.
7. *Hirsch J. G.*: The phagocytic cell in host resistance. A perspective summation. In: Bellanti, J. A. & Dayton D. H. (Ed.): The phagocytic cell in host resistance. Raven Press, New York, 1975 p. 333–340.
8. *Hirsch J. G.*: Phagocytosis. Ann. Rev. Microbiol. *19* 339–350 1965.
9. *Jenkin C. R.*: The effect of opsonins on intracellular survival of bacteria. Brit. J. exp. Path. *44* 57 1963.
10. *Mainland D.*: Elementary medical statistics. 2. ed. W. B. Saunders Company. Philadelphia and London, 1964.
11. *Rowley D.*: Bactericidal activity of macrophages *in vitro* against *Escherichia coli*. Nature *181* 1738–1739 1958.
12. *Solberg C. O.*: Enhanced susceptibility to infection. A new method for the evaluation of neutrophil granulocyte functions. Acta path. microbiol. scand. Sect. B *80* 10–18, 1972.
13. *Stossel Th. P.*: Phagocytosis. N. Engl. J. Med. *290* 774–780 1974.
14. *Stossel Th. P.*: Phagocytosis. N. Engl. J. Med. *290* 833–839 1974.
15. *Talstad I.*: The relationship between phagocytosis of polystyrene latex particles by polymorphonuclear leucocytes (PML) and aggregation of PML. Scand. J. Haemat. *9* 516–523 1972.
16. *Trippestad A. & Midtvedt T.*: Phagocytosis of ^{32}P labelled *E. coli* by rat polymorphonuclear leucocytes. Evaluation of a method. Acta path. microbiol. scand. *74* 259–273 1968.

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TUMOR Fc RECEPTORS AND TUMOR ASSOCIATED IMMUNOGLOBULINS

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Tönder O., Krishnan, E. C., Jewell, W. R., Morse P. A., Jr. & Humphrey L. J. Tumor Fc receptors and tumor-associated immunoglobulins. Acta path. microbiol. scand. Sect. C, 84 105–111 1976.

Tissue sections and cell suspensions from ten malignant tumors were tested for Fc receptors using sheep erythrocytes sensitized by rabbit IgG antibodies. Surface bound IgG on cells from the same tumors were estimated using an antiglobulin consumption test with ^{125}I labelled human IgG as reference. The amount of IgG present per 10^6 cells varied from <100 ng to approximately 600 ng. When these amounts of IgG were plotted against the Fc receptor activity of corresponding tumors, seven of the tumors were distributed along a line showing an inverse linear relationship; i.e. tumors with large amounts of IgG on their cell surfaces had the lowest Fc reactivity and vice versa. Cells from three of the tumors had lower amounts of IgG on their surface than expected from this relationship. However the lack of correlation could be explained by the focal distribution of the Fc positive tissue within non-reactive tissue. The cells from these areas presumably carry less IgG on their surface and thereby reduce the quantity of IgG calculated per 10^6 cells. Prolonged washing of tumor sections resulted in stronger Fc receptor activity and correspondingly washed cells had lower amount of IgG on their surface. Presumably the Fc receptor can bind IgG in vivo.

Key words Fc receptor tumor-associated Ig.

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Evidence has been presented that many human malignant tissues possess a receptor for the Fc region of IgG particularly recognizing Fc in antigen-antibody complexes in preference to IgG alone (15 16). It is also well established that many malignant tumors contain significant amounts of immunoglobulins (8, 14 18) and the evidence for actual coating of tumor cells in vivo by immunoglobulins is convincing (reviewed in 18).

The mechanism of binding of immunoglobulin may be a specific antigen-antibody reaction, i.e. the Fab portion of antibody reacts with antigens on the tumor cell surface. Several investigators have speculated that this reaction is responsible for the enhancement of tumor growth (6 12 18). However our data (15) suggest that immunoglobulin may also be bound in vivo by the Fc portion in a non-specific manner

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mediated by the Fc receptor present in the tissue. Fc receptors coated *in vivo* would not be available for immediate *in vitro* detection. Experiments were designed to test this hypothesis.

Data presented compare the amounts of immunoglobulin bound *in vivo* with the *in vitro* Fc reactivity of selected human malignant tumors, using tissue sections and cells in suspension. Since the tumor Fc receptor has affinity mainly for IgG (16), these investigations deal with tumor associated IgG only.

MATERIALS AND METHODS

Tissues

Specimens from malignant tumors were obtained from tissue removed during surgery. Specimens from normal liver and spleen were kindly provided by the Department of Pathology. Tissue not used immediately was frozen in phosphate buffered saline, pH 7.2 (PBS) and wrapped in plastic bags to be stored at −70 C. Cryostat sections of tissue were prepared and handled as described earlier (15, 16).

Histological investigations and classification of tumors were routinely performed in the Department of Pathology. Consecutive sections of frozen tissue, in addition to those used in the tests, were stained with hematoxylin and eosin in order to provide histopathological confirmation of the tissue under study.

Cell Suspensions

Fresh tissue was thoroughly minced with iris scissors and suspended in PBS. The suspension was centrifuged at 100 × g for 1 min to sediment the coarser particles, and the supernatant was saved. The cells were sedimented by centrifugation at 650 × g for 5 min, washed 3 times in PBS and resuspended to approximately 2 × 10⁶ cells/ml. For some experiments aliquots of cells were saved before each washing, and washing was continued up to five times.

Serum and Serum Fractions

Sera from 25 healthy blood donors were pooled. Immunoglobulin G was isolated by exclusion chromatography according to the method described by *Flodin & Killander* (4). It was further purified by treating twice with DEAE Sephadex at pH 6.5 (1). The purified preparation produced a single IgG line in agar diffusion and immunoelectrophoresis when tested against antisera to whole human serum, and this line gave identity with the line produced by a commercial antiserum to IgG.

Antiserum to human IgG was produced by injecting rabbits subcutaneously with 200 µg in complete Freund's adjuvant at multiple sites every other week for 10 weeks. The rabbits were bled 10 days after the last injection. The antiserum gave only an IgG line against human serum in agar diffusion and immunoelectrophoresis. Antiserum specific to human IgG was also purchased from Hyland, Costa Mesa, California, USA. Before use the antisera were ultracentrifuged in order to remove complexes caused by storage.

Preparation of Labelled IgG

The proteins were labelled with ^{125}I according to the method described by *Hunter & Greenwood* (7). The average labelling was 0.5 to 1 atom of iodine per molecule of protein. The labelled preparations were dialysed for 72 hours against PBS and then passed through 1 cm × 10 cm G-50 Sephadex columns.

Radioimmunoassay for Quantitation of IgG

The method has been described in detail elsewhere (8). The optimum ratio of antiserum to labelled and unlabelled human IgG was determined by testing a series of increasing dilutions of antiserum against 400 ng of labelled IgG. The dilution of antiserum required to precipitate the maximum amount of labelled IgG at 35 per cent saturation of ammonium sulfate was determined. The proper saturation of ammonium sulfate needed to separate bound and unbound IgG was determined as described by *Farr* (2). Diluent was normal rabbit serum 1 in 50 in 0.05 borate buffer pH 8.4. To obtain the standard curve, 0 to 800 ng of unlabelled human IgG were added to rabbit anti-human IgG of appropriate dilution as determined above. The mixtures were incubated at 4 C for 24 h. 400 ng of labelled IgG was added and incubation was continued for another 24 h at 4 C. Ammonium sulfate was added to make up a final volume of 5 ml with saturation 35 per cent. After centrifugation at 1 100 × g for 30 min, 1 ml of the supernatant and the rest including the precipitate, were counted separately. The percent precipitated ^{125}I IgG was calculated from the formula

$$^{125}\text{I IgG precipitated} = \frac{R - 4S}{(R + S)} \quad \text{where } R =$$

cpm in the rest, and S = cpm in 1 ml supernatant.

Percent of ^{125}I IgG precipitated was determined for different amounts of unlabelled IgG and a standard curve was obtained (Fig. 1).

To determine the quantity of IgG on the cells, 10⁶ washed cells were incubated in triplicate for 24 h with antiserum of the same dilution as used to obtain the standard curve. After incubation, the

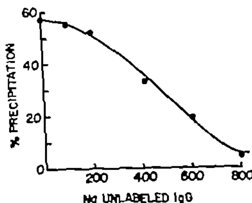


Fig 1 Standard curve for IgG quantitation. Per cent labelled IgG precipitated after preincubation with various amounts of unlabelled IgG

cells were removed by centrifugation, 400 ng ^{125}I IgG was added to the supernatant and the mixture was incubated for another 24 h. The percent precipitation was determined as described above. The amount of bound IgG was expressed in terms of ng/10^6 cells by referring to the standard curve

Detection of Fc Receptors

Sheep erythrocytes (E) sensitized with various amounts of corresponding rabbit IgG antibodies (A) expressed as agglutinating units were prepared as described earlier (15 16) One agglutinating unit is defined as the amount of the highest dilution of antiserum which agglutinates an equal amount of 1 per cent suspension of E.

The techniques for detection of Fc receptors in tissue sections and on single cells in suspensions have also been described (15, 16) In short, cryostat sections of frozen tissue on large cover glasses were incubated at 20 C (room temperature) in wet chambers with the EA applied using inverted microculture slides with a single concavity allowing the EA (suspended in PBS containing 2 per cent NaCl) to settle on the cover glass. After 30 min at 20 C, the slides were turned coverglass up, and left for detachment of EA from the glass and non-reactive tissue. The degree of hemadsorption (3+ 2+ 1+) was recorded microscopically when the glass around the tissue was free of erythrocytes.

One drop of cells in suspension (approximately 2×10^6 cells/ml) was mixed in glass tubes with 1 drop of the proper EA suspension containing approx 2×10^8 erythrocytes/ml (1 per cent suspension) The mixtures were left at 20° C for 2 h. The sedimented cells were then gently resuspended and the percentage of rosette forming cells determined using phase contrast microscopy at 450×

For morphological studies, smears were stained with methylgreen and pyronine.

EXPERIMENTS AND RESULTS

Fc Receptors in Tissue and on Cells

For complete experiments only those tumors could be used which gave good single cell suspensions after mechanical disintegration. Ten tumors were selected according to this criterion. Trypsin treatment was avoided because it would destroy bound IgG The results obtained with these tumors in tests with sections and EA are shown in Table I The patterns of reactions are also given, as diffuse or focal types, *i.e.* most of the entire section is covered in contrast to larger or smaller areas.

The ten tumors showed very different activity without any relation to type of tumors. Cells in suspension from the tumors which adsorbed EA in diffuse patterns formed rosettes with EA in percentages which corresponded well with the results obtained with sections, while the percent rosettes with cells from the tumors showing focal reactions was very low (<5 per cent) The results obtained with sections are therefore used.

IgG on the Cells

Cells in suspension from each of the tumors were tested for presence of IgG bound to the cell surface. The mean values of duplicate determinations are given in Table 1 and in Fig 2 they are plotted against Fc reactivity in sections of corresponding tumors. An inverse relationship was observed, *i.e.* with high amounts of IgG on the surface the Fc reactivity was low while with low amounts of IgG bound to the cells, the Fc activity was pronounced. The tumors which fall outside this relationship were tumors showing focal reactions with few and/or small foci mixed with histologically non-malignant tissue. These results indicate that a significant relationship exists between bound immunoglobulins and availability of Fc receptor sites in/on the malignant cells.

TABLE 1 *Fc Receptor Activity in Tumor Tissue Sections Measured by Adsorption of EA and Presence of IgG on Dispersed Cells*

Type of tissue		Test with tissue sections										
		EA and no. of agglutinating units of A									Patterns of ads. EA	IgG ng/10⁶ cells
		4	2	1	1/2	1/4	1/8	1/16	1/32	E*		
Melanoma	T 4	3+	3+	2+‡	–	–	–	–	–	–	Focal	100
	T 7	3+	3+	3+	*2+*	±	–	–	–	–	Diffuse	574
	T 18	2+	3+	3+	*2+*	1+	±	±	±	±	Diffuse	600
Carcinoma of												
Breast	T 15	2+	2+	2+	*1+*	±	–	–	–	–	Focal	590
	T 40	3+	3+	3+	3+	2+	*1+*	–	–	–	Diffuse	<100
	T 53	3+	2+	1+	*1+*	–	–	–	–	–	Diffuse + Focal	400
Thyroid	T 41	3+	2+	*1+*	–	–	–	–	–	–	Focal	285
Colon	T 37	2+	2+	1+	*1+*	–	–	–	–	–	Focal	300
Bladder	T 10	3+	3+	2+	2+	2+	*1+*	–	–	–	Diffuse	350
Leiomyosarcoma	T 8	3+	3+	3+	3+	3+	2+	2+	*1+*	–	Diffuse	<100

* Controls with unsensitized E.
‡ Numbers in italics considered endpoints.

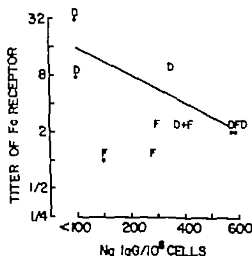


Fig. 2 Amount of IgG in ng/10^6 cells on cells in suspensions plotted against Fc receptor activity in sections of the same tumors. D Diffuse attachment of EA to tissue sections. F Focal attachment of EA to tissue sections. The line represents the median values of tumors which gave D attachment.

Effect of Washing on Fc Receptor Activity

Since the Fc attachment is relatively weak, it was predicted that thorough washing of tissue would increase the receptor activity. Sections of two tumors, spleen and liver were kept in PBS for a period of three days at 4° C and 20° C, and sections were removed after various length of time and tested against EA.

The activity of the two tumors increased after washing up to 24 h at both temperatures, and remained unchanged from this time on (Table 2). There was not only an increase in titer of Fc receptor activity but with each reactive EA the tissue was more heavily covered compared to unwashed tissue. Experiments using rosette formation with cells in suspension confirmed these results. In addition, cells in suspension, washed five times and tested for presence of IgG after each wash, showed a noticeable decrease in IgG concentrations, *e.g.* from approximately 800 and 600 ng after one washing to 574 and 350 ng/10^6 cells after three washings. Further washing showed little change.

The Fc receptor activities of liver and spleen did not change significantly during the washing procedure.

DISCUSSION

The results presented confirm previous observations that human malignant tissues possess receptors for IgG Fc as demonstrated by adsorption of EA (15, 16) and that the cells are coated *in vivo* with IgG (8, 18). In addition, they indicate an inverse relationship between available Fc receptors on/in the cells and presence of IgG on the cell surface, i.e. tumors with the largest amounts of IgG showed the lowest reactivity with EA. Al-

TABLE 2. *Fc Receptor Activity in Tissue Sections Measured by Adsorption of EA. Effect of Prolonged Washing of the Sections in PBS*

Tissue	EA and no. of agglutinating units of A						
	2	1	1/2	1/4	1/8	1/16	1/32
T 40	3+	3+	3+	2+F*	1+F	–	–
washed	3+	3+	3+	3+D‡	2+D	1+F	–
T42	3+	3+F	2+F	–	–	–	–
washed	3+	3+D	3+D	2+F	1+F	–	–
Spleen	3+	2+	2+	1+	–	–	–
washed	3+	2+	2+	1+	–	–	–
Liver	3+	3+	2+F	1+F	–		–
washed	3+	3+	2+F	1+F	–	–	–

F Focal attachment of EA to tissue sections.
‡ D Diffuse attachment.

though the relationship was not ideal with all tumors as seen from Fig 2 those tumors deviating from this relationship also showed other differences. Section of tumors which gave diffuse patterns of Fc receptor activity showed very little normal tissue present, and therefore the cell suspensions of these tumors would represent a relatively homogenous cell population. On the other hand the tumors presenting a focal type of receptor activity yielded greater amounts histologically normal tissue. Cell suspensions prepared from these tumors will have a large percentage of cells carrying less IgG on their surface, thereby reducing the quantity of IgG calculated per 10^6 cells. Such tumors would then give lower average IgG concentration on the cells than predicted from the Fc receptor activity within the foci

The technique used for quantitation of IgG on the cells has a high sensitivity and al thoug controls showed that the antiserum was specific for IgG cross reactions with other Ig classes on the cells may have influenced the results to some extent. Yet the values obtained with this technique for IgG on human peripheral lymphocytes (8) corresponded well with those reported by others (13) What is more important for the evaluation of the results obtained with tumor cells is that IgC anti IgG may attach to the Fc receptors which are not coated *in vivo* and unspecifically absorb the anti IgG thereby giving higher values for IgG than actually is present. However the results of other experiments (*Krishnan et al* unpublished results) indicate that although there is binding of added anti IgG by the Fc fragment, this accounts for a minor proportion of the total consumption. (Since the antiglobulin serum was used unabsorbed it should not contain any antigen-antibody complexes which might interfere with the Fc receptor) Some tumors carrying relatively low amounts of *in vivo* bound IgG may have more Fc receptor sites with higher avidity available These tumors therefore give higher values of IgG on the surface than actually is present. This bias by the technique can be overcome by using the $F(ab')_2$ fragment of the antibody IgG instead of the whole molecule, and experiments are in progress to modify the technique using these fragments.

Our results do not offer any direct evidence that IgG binds to Fc receptors *in vivo* and thereby blocks the *in vitro* reaction with EA. But the data suggest that this mechanism is operating The decrease in IgG content parallel to the increase in Fc reactivity following washing of the tissues is strong evidence supporting this conclusion. IgG (complexed or not) may bind *in vivo* to the most avid Fc receptors, leaving those with lower avidity and which react preferably with antigen-antibody complexes available for *in vitro* testing Further experiments with eluates of tumor cells and EA prepared using autologous IgG are needed to test this possibility

The binding of IgG by the Fc fragment is a property which is shared by many cells such as lymphocytes, monocytes, polymorphonuclear leucocytes, and malignant cells (see 15) The present results give no answer to the question whether the Fc receptor bearing cells actually are the malignant cells, or infiltrating host cells or both. Evidences have recently been presented indicating that many or all of the Fc receptor bearing cells in several murine tumors are of host origin (mainly macrophages) (10) Detailed studies are needed to reveal whether this is the case with human malignant tumors.

However irrespective of whether the target cells or the "effector cells" are involved the presence in malignant tissues of Fc receptor bearing cells may have important biological implications in tumor immunology and the mechanisms of binding of tumor associated Ig need to be retested and reevaluated The Fc receptor may well be the site of attachment for the so-called "blocking antibodies which evidently in some instances are antigen antibody complexes (9 12) particularly since high serum concentrations (20 per cent) are often used for their detection (5 6 17) That eluates of tumor tissues contain large amounts of Ig (14 18) which may combine preferably with tumor tissues, even

with a certain degree of specificity (5 11) is no proof that the binding is of antigen-antibody type. Experiments with fragments of the isolated IgG are necessary to determine the type of binding involved.

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REFERENCES

1 *Benedict, J S Laffin R. J & Bardawil, W A* A preparatn method for the separation of 7S gamma globulin from human serum. Arch. Biochem *106* 514–521 1964

2 *Farr R. S* A qualitatv immunochemical measure of the primary interaction between I BSA and antibody J Infect. Dis. *103* 239–262 1958.

3 *Fish, F Witz I P & Klein, G* Tumour bound immunoglobulins The fate of immunoglobulin disappearing from the surface of coated tumour cells. Clin. exp. Immunol *16* 355–365 1974

4 *Flodin P & Killander J* Fractionation of human serum proteins by gel filtration. Biochim biophys. Acta (Amst) *63* 403–410, 1962

5 *Hellström, I & Hellström, K E.* Studies on cellular immunity and its serum-mediated inhibition in Moloney-virus-induced mouse sarcomas Int. J Cancer *4* 587–600 1969

6 *Hellström I Sjögren H O Warner G & Hellström K E* Blocking of cell-mediated tumor immunity by sera from patients with growing neoplasms. Int. J Cancer *7* 226–237 1971

7 *Hunter W M & Greenwood F C.* Preparation of iodine-131 labelled human growth hormone of high specific activity Nature (Lond.) *194* 495–496 1962.

8 *Jewell, W R. & Krishnan E.C* Quantitatn radioimmunoassay of immunoglobulins on the surface of human tumor cells. J Surg. Res. *16* 424–427 1974

9. *J w D G & Seshadri R.* Circulating immune complexes in human neuroblastoma: direct assay and role in blocking specific cellular immunity Int. J Cancer *13*: 824 838, 1974

10. *Kerbel, R. S., Pross H F & Elliott E. V.* Origin and partial characterization of Fc receptor-bearing cells found within experimental carcinomas and sarcomas. Int. J Cancer *15* 918–932, 1975

11 *Ran M Fish F., Witz I P & Klein G.* Tumourbound immunoglobulins. The *in vitro* disappearance of immunoglobulin from the surface of coated tumour cells, and some properties of released components. Clin. exp. Immunol. *16* 335–353 1974

12. *Sjögren H O Hellström I., Bansal S C. & Hellström K E.:* Suggestive evidence that the "blocking antibodies of tumor-bearing individuals may be antigen-antibody complexes. Proc. nat. Acad. Sci. (Wash.) *68* 1372 1375 1971

13 *Smith R. S., Longmire R L Reid R T & Farr R S.* The measurement of immunoglobulin associated with human peripheral lymphocytes. J Immunol. *104* 367–376 1970

14 *Thunold S Tönder O & Larsen O.:* Immunoglobulins in eluates of malignant human tumours. Acta path. microbiol. scand. Sect. A. Suppl. *236* 97–100 1973

15 *Tönder O., Morse P A. J & Humphrey L. J.* Similarities of Fc receptors in human malignant tissue and normal lymphoid tissue J Immunol. *113* 1162 1169 1974

16. *Tönder O & Thunold S.* Receptors for immunoglobulin Fc in human malignant tissues. Scand. J Immunol. *2* 207–215 1973

17 *Vánky F., Stjernswärd J., Klein G Steiner L. & Lindberg, L.* Tumor-associated specificity of serum-mediated inhibition of lymphocyte stimulation by autochthonous human tumors. J nat. Cancer Inst. *51* 25–32, 1973

18. *Witz, I P* The biological significance of tumor-bound immunoglobulins. Curr Top. Microbiol. Immunol. *61*: 151–171 1973

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INFLUENCE OF ANTIBODIES AND THERMOLABILE SERUM FACTORS ON THE BACTERICIDAL ACTIVITY OF HUMAN NEUTROPHIL GRANULOCYTES

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Solberg C. O., Christie, K. E. Larsen, B & Tonder O *Influence of antibodies and thermolabile serum factors on the bactericidal activity of human neutrophil granulocytes.* Acta path. microbiol scand Sect. C 84 112-118 1976

The influence of serum antibodies and thermolabile serum factors on the intracellular killing of *Staphylococcus aureus* by neutrophil granulocytes has been examined using a method which facilitates a precise *in vitro* evaluation of the phagocytic and bactericidal activities of human polymorphonuclear leucocytes. The bactericidal activity of the granulocytes was significantly less pronounced in the presence of serum absorbed with *Staph. aureus* or inactivated at 56 C for 30 minutes than in the presence of untreated serum. Specific antibodies seemed to stimulate the intracellular killing of bacteria more than thermolabile serum factors.

Key words Granulocytes phagocytosis intracellular killing influence of serum factors.

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Phagocytosis and intracellular killing of bacteria are important physiological functions of neutrophil granulocytes. It is well known that antibodies and heat labile serum factors markedly enhance the phagocytic activity of the granulocytes (4 5 6 15) Whether these factors also influence the intracellular killing of bacteria is poorly understood.

If this problem is to be studied, analyses of the phagocytic as well as the bactericidal activities of the granulocytes are required. However studies of the interactions of bacteria and granulocytes have usually been confined to the ingestion phase or phagocytosis and little attention has been paid to the dynamics of the intracellular phase (for review see 1 3 5) In previous investigations, a method has been developed which facilitates a precise *in vitro* evaluation of the phagocytic and the bactericidal activities of the granulocytes (7 9 11) Using this method, it was demonstrated that normal human serum by way of the granulocytes markedly enhanced not only the phagocytosis but also the intracellular killing of bacteria (10)

The present study is concerned with the influence of serum antibodies and heat labile serum factors on the intracellular killing of bacteria by neutrophil granulocytes

MATERIALS AND METHODS

Leucocytes

Leucocytes obtained from normal individuals by Isopaque /dextran sedimentation of heparinized enous blood (10 units heparin per ml blood) were twice washed in heparinized saline (1 unit heparin per ml saline) by centrifugation at 500 g for 5 minutes (7)

A differential count was made and the cells were resuspended to 10^7 neutrophils per ml in Hanks balanced salt solution containing 0.1 per cent gelatin. The functional integrety of the isolated neutrophils remained intact as measured by latex particle phagocytosis and 95 to 99 per cent resisted staining with trypan blue. Eosinophil granulocyte contamination in 50 consecutive specimens aried from 0 to 7 per cent (mean 3 per cent) basophil granulocyte contamination from 0 to 2 per cent (mean 1 per cent) and lymphocyte-monocyte contamination from 12 to 23 per cent (mean 17 per cent) Small amounts of autologous serum, though less than 0 02 per cent, re mained.

Bacteria

Staphylococcus aureus "Oxford" (Heatley strain, obtained from the National Collection of Type Cultures, Colindale, London, 1958) was used as the test organism (7) The bacteria were cultured overnight in Penassay broth (Difco) twice washed in 0 45 per cent saline and suspended in Hanks balanced salt solution to an optical density of 0 6 at 620 nm in Beckman spectrophotometer (7) This suspension was diluted in Hanks balanced salt solution containing 0.1 per cent gelatin to a concentration of 8-12 × 10^7 colony forming units per ml.

Serum

Fresh, pooled normal serum from six adults was stored at 50 C in 1 ml aliquots. Immediately before each experiment, serum was thawed and added to Hanks balanced salt solution containing 0 1 per cent gelatin to make appropriate serum dilutions. All experiments in the present study were per formed with the same pool of serum.

Heat labile serum factors were inactivated by incubation of freshly thawed normal serum for 30 minutes t 56 C.

With ew to absorption of antibodies in the serum to *St ph. aureus* 4 ml serum diluted 1:5 in Hanks balanced salt solution were mixed with approximately 2 ml of packed bacteria (harvest from 10 Penassay agar plates) The bacteria had been twice washed in 0 45 per cent saline by centrifugation at 5000 g for 20 minutes at 4 C. The mixture was incubated for 45 minutes at 4 C, centrifuged at 5000 g for 20 minutes and the supernatant (bsorbed serum) stored in 1 ml aliquots. In slide and tube agglutination tests, the absorbed serum gave only trace agglutination in the 1:5 dilution in contrast to unabsorbed serum which agglutinated in dilutions up to 1:512.

Test Procedure

0.5 ml leucocyte suspension, 0.1 ml bacteria suspension, and 0.4 ml diluted serum were added to 12 × 75 mm disposable plastic tubes. This provided about 2 bacteria per granulocyte and final serum concentrations as those indicated in the fig ures (vide infra) The tubes were incubated at 37° C using end over end rotation to promote contact between bacteria and leucocytes. Samples were removed periodically for determinations of the total number of iable bacteria and the number of iable intracellular bacteria.

The total number of viable bacteria was deter mined after osmotic disruption of the leucocytes by adding 0 01 ml leucocyte-bacteria suspension to 1 ml distilled w ter Quantitation of iable bacteria was made from appropriate dilutions of this suspension using standard pour-plate technique and Penassay agar (Difco)

The number of viable intracellular bacteria was determined as described earlier (7): 0.01 ml of the leucocyte-bacteria suspension and 1 ml Hanks balanced salt solution containing 0.1 per cent gelatin, 500 μg streptomycin, 500 units penicillin G and 2 mg phenylbutazone were incubated at 37 C for 15 minutes and centrifuged for 10 minutes at 500 g The cellular pellet was twice washed in 5 ml Hanks balanced salt solution by centrifugation at 500 g for 10 minutes and resuspended in 1 ml distilled water to allow osmotic disruption of the leucocytes to occur Quantitation of iable bacteria was made by the standard pour-plate technique.

The bactericidal capacity of the granulocytes is proportional to the total number of bacteria killed and inversely proportional to the total number of iable bacteria or number of iable intracellular bacteria (7) The number of bacteria phagocytized equals the number of viable intracellular bacteria plus the number of bacteria killed (7)

Controls

Controls consisting of mixtures of bacteria and serum without leucocytes served to detect any direct bactericidal effect of the serum or serum fac tors, and mixtures of leucocytes and bacteria incubated without rotation served to detect any extracellular bactericidal activity caused, for example, by enzymes liberated from damaged granulocytes. N reduction in the number of viable bacteria was observed in these control tests during 2 hours incubation.

Statistical Method

The Wilcoxon-test for two samples was used

RESULTS

Influence of Inactivated Serum

Leucocyte bacteria suspensions were incubated with inactivated and normal serum and the total number of viable bacteria and the number of viable intracellular bacteria were determined.

In the tests containing normal serum at a final concentration of 1 per cent or more phagocytosis and killing of bacteria occurred rapidly (Figs. 1 and 2) In the tests with inactivated serum the reduction in the total number of viable bacteria was significantly less pronounced and the number of viable intracellular bacteria increased slowly during the early phase of incubation later it remained higher indicating that the phagocytosis and to a minor degree, the bactericidal activity of the granulocytes were reduced.

Influence of Absorbed Serum

A test similar to that described above was performed using absorbed and normal serum.

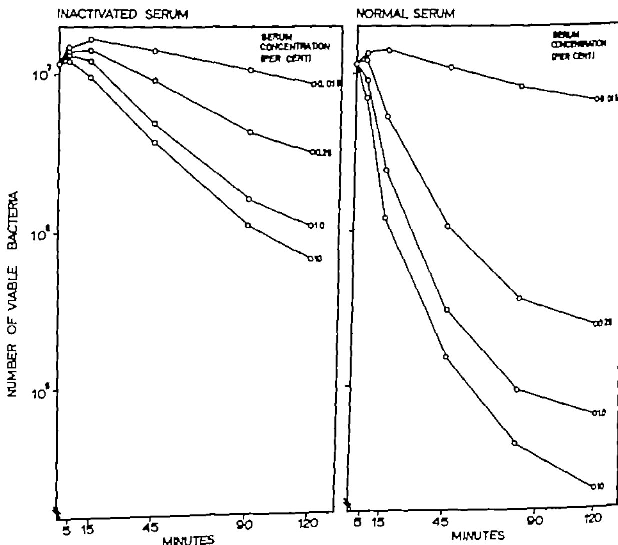


Fig. 1 Total number of viable bacteria during incubation of granulocyte-bacteria suspensions with inactivated and normal serum (mean of four experiments) The differences between 0.25 1.0 and 10 per cent inactivated and normal serum at 45 90 and 120 minutes were significant ($p \leq 0.025$)

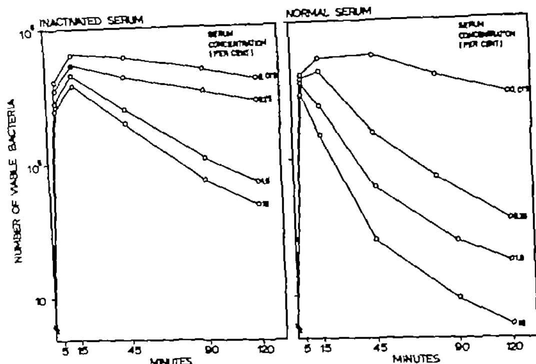


Fig 2 Number of viable intracellular bacteria during incubation of granulocyte-bacteria suspensions with inactivated and normal serum (mean of four experiments) The differences between 0.25 1.0 and 10 per cent inactivated and normal serum at 45 90 and 120 minutes were significant ($p \leq 0.025$)

The phagocytic and bactericidal activities of the granulocytes were significantly less pronounced in the presence of absorbed serum than in the presence of normal serum (Fig 3) If inactivated absorbed serum was added to leucocyte bacteria suspensions, the phagocytic and bactericidal activities of the granulocytes would be as poor as that to be seen if Hanks balanced salt solution had been used (10) Conversely if absorbed serum was added to leucocyte-bacteria suspensions containing inactivated serum, the phagocytic and bactericidal activities of the granulocytes would increase to approximately the same level as that to be seen if normal serum had been used.

DISCUSSION

Many of the studies of interactions of bacteria and granulocytes have been confined to phagocytosis and little attention has been paid to the dynamics of the intracellular phase (1 3, 5) This has partly been due to technical difficulties. A major problem has remained namely the separation of extracellular and intracellular bacteria in an *in vitro* phagocytic system in order to evaluate the intracellular bactericidal processes (2, 7 8, 9 12) By our method, extracellular bacteria are effectively controlled by antibiotics which do not inactivate intracellular bacteria (9) Killing of extracellular bacteria by antibiotics takes, however 10–15 minutes. During this period, inactivation of intracellular bacteria by granulocyte enzymes may significantly obscure the results (9) Thus, inhibition of the bactericidal activity of the granulocytes by phenylbutazone is a prerequisite for the determination of the number of viable intracellular bacteria while extracellular killing by antibiotics takes place. Accordingly

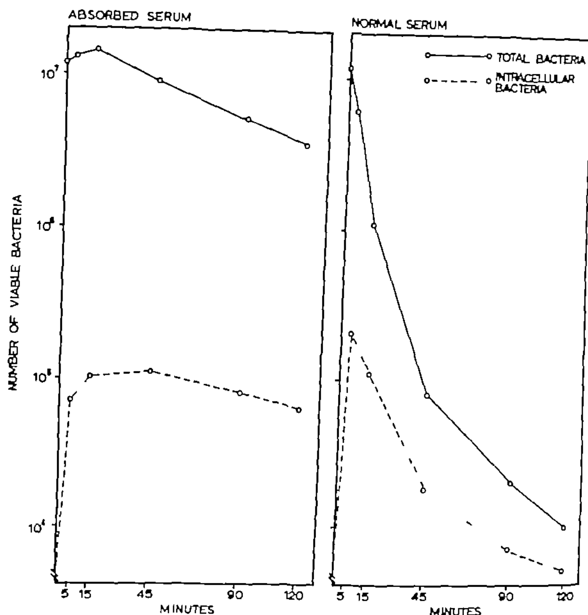


Fig 3 Viable bacterial counts during incubation of granulocyte bacteria suspensions with absorbed and normal serum at concentrations of 10 per cent (mean of four experiments) The differences between absorbed and normal serum at 45 90 and 120 minutes were significant ($p \leq 0.025$)

the essence of our method is the combined use of phenylbutazone for the inhibition of intracellular killing of bacteria and antibiotics for the control of extracellular bacteria

In the tests using absorbed serum large numbers of bacteria remained in extracellular positions. However significant numbers of bacteria were phagocytized and killed indicating either that phagocytosis and intracellular killing of bacteria may take place in the absence of specific antibodies or that small amounts of antibodies were still present in the absorbed serum or leucocyte suspension. Agglutination tests demonstrated that the amounts of specific antibodies in absorbed serum were too small to promote the phagocytosis and intracellular killing of *Staph aureus* (10) Furthermore the small amount of serum in the leucocyte suspension can also be considered to be negligible since the phagocytic and bactericidal activities of the granulocytes were significantly less pronounced in the presence of Hanks balanced salt solution than in the presence of absorbed se-

rum (10) However when the absorbed serum and the leucocyte suspension are added together as in the present test system, the amount of antibodies may increase to a concentration of such magnitude that complement components will be activated and significant phagocytosis and intracellular killing of bacteria will be promoted. On the other hand, it has been demonstrated that Fc-binding of nonspecific antibodies to *Staph. aureus* may stimulate the phagocytosis of the bacteria by human neutrophils, probably by activating complement components (13 14). It remains to be determined whether the phagocytosis and intracellular killing of bacteria by the granulocytes in the presence of absorbed serum is due to small amounts of specific antibodies still present in the test suspension or to Fc-binding of nonspecific antibodies to the bacteria.

The phagocytic and bactericidal activities of the granulocytes were significantly less pronounced in the presence of inactivated serum than in the presence of normal serum. Even after incubation for two hours, the total number of viable bacteria and the number of viable intracellular bacteria were markedly higher in the tests with inactivated serum. On the other hand, both phagocytosis and intracellular killing of bacteria significantly increased when inactivated serum was added to the leucocyte-bacteria suspensions instead of absorbed serum, indicating that the stimulating effect of specific antibodies on the bactericidal activity of the granulocytes was superior to that of heat labile serum factors.

The reason why the bactericidal activity of the granulocytes was reduced in the absence of specific antibodies or heat labile serum factors remains unknown. However if significant phagocytosis is to occur attachment of sensitized bacteria to the phagocytic cell membrane by the Fc part of specific antibody molecules is required (Fc-receptor) (4 5 6, 15). Heat labile serum factors, mainly complement components, probably stimulate phagocytosis by facilitating the attachment of sensitized bacteria to the phagocyte membrane (C-receptor). As soon as attachment occurs, the bacterium will be surrounded by the leucocyte cell membrane which is form ing a phagocytic vacuole into which bactericidal enzymes are released. The attachment of sensitized bacteria by specific antibodies and heat labile serum factors probably acts as a stimulus for the release of these enzymes. This would explain the markedly reduced bactericidal activity of the granulocytes in the absence of specific antibodies or heat labile serum factors.

REFERENCES

1. *Brandt L.*, Studies on the phagocytic activity of neutrophilic leucocytes. Scand. J Haemat. Suppl. 4, 1967
2. *Craig C P & Suter E* Extracellular factors influencing staphylocidal capacity of human polymorphonuclear leucocytes. J Immunol. 97 287-296, 1966
3. *Hirsch J G* Neutrophil and eosinophil leucocytes. *In* Zweifach, B. W Grant, L. & McCluskey R. T (Eds.) The inflammatory process. Academic Press, Inc., New York, 1965 p 45-280.
4. *Messner R. P & Jelinek J.*. Receptors for human γG globulin on human neutrophils. J Clin. Invest. 49 2165-2171 1970.
5. *Quie P G.*: Disorders of phagocyte function. Year Book Medical Publishers, Inc., Chicago, 1972.
6. *Quie P G. Messner R P & Williams R. C., Jr* Phagocytosis in subacute bacterial endocarditis. Localization of the primary opsonic site to Fc fragment. J Exper Med. *128* 553-570 1968.
7. *Solberg*, C O Enhanced susceptibility to infection. A new method for the evaluation of neutrophil granulocyte functions. Acta path. microbiol. scand. Sect. B, *80* 10-18 1972.
8. *Solberg*, C O., Evaluation of neutrophil granulocyte functions. Acta path. microbiol. scand. Sect. B, 80 559-563 1972.
9. *Solberg*, C O., Protection of phagocytosed bacteria against antibiotics A new method for the evaluation of neutrophil granulocyte functions. Acta med. scand. *191* 383-387 1972.
10. *Solberg C O & Hellum K. B.* Influence of serum on the bactericidal activity of neutrophil granulocytes. Acta path. microbiol. scand. Sect. B, *81* 621-626 1973
11. *Solberg, C O & Hellum K. B.* Neutrophil granulocyte function in bacterial infections Lancet *II* 727-730 1972.
12. *Sands J B Booth R. R. & Orner, N* Eva-

luation of phagocytic activity by ingestion of labeled bacteria J Infect. Dis. *123* 93–96 1971

13 *van Oss C J & Stinson M W* Immunoglobulins as specific opsonins. I The influence of polyclonal and monoclonal immunoglobulins on in vitro phagocytosis of latex particles and staphylococci by human neutrophils. J Reticuloendothel Soc. *8* 397–406 1970

14 *van Oss C J Woeppel M S & Marquart, S E* Immunoglobulins as specific opsonins. III The opsonizing power of fragments of polyclonal and monoclonal immunoglobulin G. J Reticuloendothel. Soc. *13* 221 230, 1973

15 *Williams R. C Jr* Opsonins in phagocytosis. In Williams, R. C., Jr & Fudenberg, H. H (Eds.) Phagocytic mechanism in health and disease Georg Thieme Publishers, Stuttgart, 1972 p. *167*–178.

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INFLUENCE OF IgG, F(ab')$_2$ AND IgM ON THE PHAGOCYTIC AND BACTERICIDAL ACTIVITIES OF HUMAN NEUTROPHIL GRANULOCYTES

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Christie, K. E., Solberg, C. O., Larsen, B., Grov, A. & Tønder, O. Influence of IgG, F(ab')$_2$ and IgM on the phagocytic and bactericidal activities of human neutrophil granulocytes. Acta path. microbiol. scand. Sect. C, 84: 119–123, 1976.

The phagocytosis and intracellular killing of *Staphylococcus aureus* by human granulocytes in the presence of immunoglobulin preparations have been examined. Isolated IgG from pooled human serum induced phagocytosis and intracellular killing of bacteria. F(ab')$_2$ fragments had no significant effect, indicating that the Fc piece of the IgG molecule is of importance not only for the phagocytosis but also for the intracellular killing of bacteria. Isolated IgM stimulated the phagocytosis to a minor extent, with no enhancement of the bactericidal activity.

Key words: Granulocytes; phagocytosis; intracellular killing; influence of immunoglobulins.

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The influence of immunoglobulins and fragments of IgG on the phagocytic activity of human leucocytes has been the subject of several investigations (1, 2, 10, 16). The immunoglobulins attach to the surface of bacteria and usually enhance the phagocytosis by human leucocytes (1, 7). This attachment is specifically mediated by the Fab piece in an antigen-antibody reaction (4, 11) and the complex attaches to receptors for the Fc piece on the cell membrane of human granulocytes (2, 3, 8, 10). Adsorption of uncomplexed immunoglobulins to the Fc receptors (cytophilic antibodies) is also of importance for the phagocytosis of bacteria. It is also claimed that immunoglobulins which are non-specifically attached to bacteria by the Fc piece can act as opsonins (16).

We have recently shown that the most important serum factors in the phagocytosis and killing of *S. aureus* by human granulocytes include specific antibodies against the bacteria (14, 15). Complement or other heat labile serum factors are also important for ingestion and intracellular killing of bacteria (5, 14).

In the present study we report further results concerning the influence of isolated IgG, IgM and pepsin digested IgG on the phagocytosis and killing of bacteria by human polymorphonuclear leucocytes.

MATERIALS AND METHODS

Leucocytes

Leucocytes were obtained from normal individuals by Isopaque/dextran sedimentation of heparinized venous blood as described previously (12)

Immunoglobulins

Human IgG (Cohn's fraction II) was purchased as a 16.5 per cent solution from AB Kabi Stockholm. The solution was dialysed against Hanks balanced salt solution (HBSS) containing 0 1 per cent gelatin and filtered through a Millipore filter (SWINNEX 13 filterunit, SXHA 013 OS 0 45 μ)

IgG was digested with pepsin (2 × crystallized Sigma Chemical Company St. Louis, Mo. USA) at an enzyme to protein ratio of 2 100 in 0 1 M acetate buffer pH 4 1 at 37 C for 16 h (9) The digestion was stopped by dialysis against phosphate buffered saline pH 7 2 (0 015 M phosphate 0 15 M NaCl) at 4 C. The digest was finally dialysed against HBSS filtered through a Millipore filter and stored at −20 C until used The antigen binding activity of the preparation did not change during digestion as the product agglutinated rabbit red cells to a titre similar to that of undigested IgG The preparation did not sensitize rabbit red cells to agglutination by rheumatoid factor indicating that the Fc portion was destroyed ($F(ab')_2$ fragments)

Human IgM was isolated from pooled normal serum by dialysation against distilled water in the cold and fractionation of the precipitate on Sephadex G-200 (2.3 × 90 cm) with phosphate buffer pH 7 2 containing 1 M NaCl as eluant. The fractions containing IgM without traceable contamination of IgG were pooled. The fractions were selected on the basis of their optical density at 280 nm, their ability to agglutinate rabbit erythrocytes before, but not after treatment with 0 2 M mercaptoethanol and their reactions with antisera against human IgG and IgM in double diffusion Membrane filtration (Sartorius membrane filter SM 13200 Göttingen) in HBSS was used to concentrate the fractions IgM in the final preparation was quantitated by single radial immunodiffusion on Tri-Partigen immunodiffusion plates (Behringwerke AG Marburg Lahn) The contamination of IgG did not exceed 0 02 mg/ml

Bacteria

Staphylococcus aureus (Heatley strain) was cultured and prepared as earlier (12) The suspensions of bacteria used contained 10 14 × 10 colony forming units per ml.

Sensitization of Bacteria

S aureus suspended in HBSS was incubated at 37 C for 30 min with various concentrations of immunoglobulins. The bacteria were then centrifuged at 10 000 *g* for 20 min, twice washed and resuspended in HBSS to a final concentration of 10–14 × 10^7 colony forming units per ml.

Test Procedure

The test procedure has previously been described in detail (12 13 14) To 0.5 ml leucocyte suspension, 0.1 ml untreated bacteria suspension was added and 0 4 ml immunoglobulin preparations diluted in HBSS 0 1 ml bacteria coated with immunoglobulins and 0 4 ml HBSS This provided 2–3 bacteria per neutrophil granulocyte. In the tests using untreated bacteria, the final concentrations of immunoglobulin will be indicated

The tubes were incubated at 37 C and samples were removed at prescribed intervals for determinations of the total number of viable bacteria and the number of viable intracellular bacteria.

Controls consisted of bacteria in HBSS mixed with granulocytes and bacteria mixed with the serum preparations to be tested in order to detect any effect of the granulocytes or serum preparations on the bacteria.

Statistical Method

The Wilcoxon-test for two samples was used.

RESULTS

Influence of IgG

Leucocyte-bacteria suspensions with 0.06 4 0 mg IgG/ml were incubated and the total number of viable bacteria and the number of viable intracellular bacteria were determined after various lengths of time Maximum phagocytosis and killing of bacteria occurred after 2 h at the final IgG concentration of 1 mg/ml (Fig 1) The number of colony forming units was reduced by 80 per cent, but still 20 per cent of the bacteria were not phagocytized

Raising the concentration of IgG did not increase the activity of the granulocytes. Whether this lack of response was due to excess of IgG molecules unspecifically blocking the attachment of sensitized bacteria to the leucocytes was further investigated Bacteria were sensitized in solutions of 0 15–10 0 mg IgG/ml and then incubated with leucocytes. Maximum phagocytosis and killing occurred at the highest concentration of IgG used

The number of colony forming units was reduced by 57 per cent but, since the control without granulocytes showed a reduction by 50 per cent, the enhancement of phagocytosis was almost negligible. There was no stimulation of intracellular killing of bacteria. Even at concentrations as low as 0.25 mg/ml, the number of viable intracellular bacteria was more than 3 times as high as that to be seen when intact IgG was used, i.e. similar to the numbers obtained in the absence of immunoglobulins. The results strongly suggest that the Fc piece of the IgG molecule is decisive not only for the phagocytosis but also for the intracellular killing of bacteria by IgG.

Bacteria sensitized with F(ab')₂ fragments were tested in the same way as described

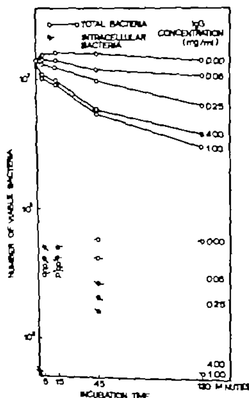


Fig. 1. Counts of viable bacteria during incubation of granulocyte-bacteria suspensions and IgG or Hanks' balanced salt solution (mean of 5 experiments). (The difference between 1 and 4 mg IgG/ml was not significant. The difference between the other groups at 120 min was significant (p≦0.025).)

(4 mg/ml) and the number of colony forming units was reduced by 95 per cent (Fig. 2). Very few bacteria escaped engulfment. However the pronounced reduction of viable bacteria was not only due to the activity of the leucocytes since controls without granulocytes showed a reduction by 40 per cent. The results indicated that the relative reduction in phagocytic activity in the presence of high amounts of IgG occurred at the level of granulocytes.

Influence of F(ab') Fragments

Leucocyte-bacteria suspensions with 0.06–4.0 mg pepsin digested IgG/ml were tested in the same way as described above for IgG.

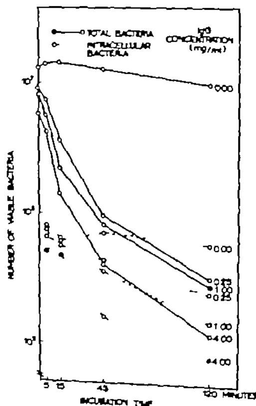


Fig. 2. Counts of viable bacteria during incubation of granulocyte suspension and bacteria sensitized with IgG (mean of 4 experiments). (The differences between the groups at 120 min were significant (p≦0.05).)

above for bacteria sensitized with intact IgG There was no enhancement of either phagocytosis or intracellular killing of bacteria

Influence of IgM

The influence on the phagocytic activity of the granulocytes of IgM at concentrations of 0 01–0.25 mg/ml was investigated At a final IgM concentration of 0 25 mg/ml, the reduction in the number of colony forming units was approximately 60 per cent. However controls showed that IgM alone in the absence of granulocytes, reduced the number of colony forming units by as much as 50 per cent. Furthermore, the number of viable intracellular bacteria was even higher than that in the controls where immunoglobulins were substituted by HBSS Accordingly, IgM did not stimulate the bactericidal activity but seemed to stimulate the phagocytic activity to a minor extent.

The phagocytic and bactericidal activities did not change when the bacteria were sensitized with IgM before addition to the leucocytes.

DISCUSSION

Two procedures were used to study the phagocytosis and intracellular killing of *S aureus* by human granulocytes in the presence of immunoglobulin preparations. In one procedure, the immunoglobulins were added to the suspension of leucocytes and bacteria in the other the bacteria were incubated with the immunoglobulins washed and then added to the leucocytes. By both procedures, isolated human IgG induced phagocytosis and intracellular killing of bacteria by human neutrophils. However the phagocytosis was more pronounced if procedure 2 was used

As the number of colony forming units in the suspensions is a crucial parameter in the tests, agglutination of the bacteria by antibodies may result in a reduction in this number without killing the bacteria. However the reduction in the number of colony forming units in a suspension of bacteria containing IgG was significantly higher in the presence of leucocytes than in the absence of leucocytes. In addition, the enhancing effect of IgG could easily be exhausted by absorption of the preparation with bacteria (14) This strongly suggests that the influence of IgG on the phagocytic and bactericidal activities of human neutrophils is due to absorption of the immunoglobulins on to the surface of the bacteria, followed by attachment of the sensitized bacteria to the cell membrane of the granulocytes (Fc receptors)

The pronounced killing of bacteria to be seen if procedure 2 was used and the relatively decreased phagocytosis at concentrations greater than 1 mg IgG/ml if procedure 1 was used indicate that non-antistaphylococcal IgG may occupy the Fc receptors on the surface of the granulocytes. The results obtained by F(ab)₂ fragments which showed no difference in phagocytosis whether procedure 1 or 2 had been used are in keeping with this suggestion.

F(ab)₂ fragments had very little if any enhancing effect on the phagocytosis, and the reduction in the number of colony forming units was apparently due to agglutination of the bacteria. The bactericidal activity was not enhanced at all. This indicates that the Fc piece of the IgG molecule is important, not only for the phagocytosis, but also for the intracellular killing of the bacteria This is in agreement with the hypothesis that the attachment of sensitized bacteria to Fc receptors on the cell membrane of the granulocytes may also influence the bactericidal activity of the granulocytes probably by acting as a stimulus by which the bactericidal enzymes may be released into the phagocytic vacuole (14 15)

Isolated human IgM had very little influence on the phagocytosis of bacteria by human neutrophils. The reduction in the number of colony forming units was mainly due to IgM alone The bactericidal activity was not increased The conclusion to be drawn on this basis is that the phagocytosis enhancing effect of IgM without complement is very low This is in accordance with the observation that monocytes and macrophages have

very few surface receptor sites for IgM (6) This may also explain the lack of bactericidal activity of the granulocytes in the presence of IgM and why complement is of such importance for phagocytosis and intracellular killing of bacteria (5 14)

REFERENCES

1. *Dossett J H Kronvall G Williams, R. C. J & Quie P G* Antiphagocytic effect of staphylococcal protein A. J Immunol. *103* 1405–1410, 1969.
2. *Fidalgo B. V & Najjar V A* The physiological role of the lymphoid system. III Leucophilic γ-globulin and the phagocytic activity of the polymorphonuclear leucocyte. Proc. Nat. Acad. Sci. U.S.A. 57 957–964 1967
3. *Fidalgo B. V & Najjar V A* The physiological role of the lymphoid system. VI The stimulatory effect of leucophilic γ-globulin (leucokinin) on the phagocytic activity of human polymorphonuclear leucocyte. Biochemistry *6* 3386–3392, 1967
4. *Lenhart N A Mudd S Yoshida, A & Li, I W* The common protein agglutinogen of *Staphylococcus aureus*. I. Distribution in international serotypes and corresponding antibody in human populations. J Immunol. *91* 771–776, 1963.
5. *Li, I W & Mudd S.* The heat-labile serum factor associated with intracellular killing of *Staphylococcus aureus*. J Immunol. *94* 852–857 1965
6. *LoBuglio A F Cotran R. S & Jandl, J H* Red cells coated with immunoglobulin G Binding and sphering by mononuclear cells in man. Science *158* 1582 1585 1967
7. *Mancher P A., Spiegelberg, H & Benacerraf B.* Studies on the mechanism of immune phagocytosis of sensitized bacteria and red cells by the reticuloendothelial system in mice. In Halpern, B. N (Ed.): Rôle du Système Réticulo-Endothélial dans Immunité Antibactérienne et Antitumorale CNRS, Paris, 1963.
8. *Messner R. P & Jelinek J.:* Receptors for human γ-G globulin on human neutrophils. J Clin. Invest. *49:* 2165–2171 1970.
9. *Nisonoff A., Wissler F C & Lipman L. N.:* Properties of the major component of a peptic digest of rabbit antibody Science *132* 1770–1771 1960.
10. *Quie P G Messner R P & Williams R. C., J.:* Phagocytosis in subacute bacterial endocarditis. Localization of the primary opsonic site to Fc fragment. J Exp. Med. *128* 553–570, 1968
11. *Shayegani M G Hisatsune K & Mudd S.* Cell wall component which affects the ability of serum to promote phagocytosis and killing of *St phylococcus au eus*. Infect. Immun. *2:* 750–756, 1970.
12. *Solberg, C O* Enhanced susceptibility to infection. A new method for the evaluation of neutrophil granulocyte functions. Acta path. microbiol. scand. Sect. B, *80* 10–18, 1972.
13. *Solberg, C. O.* Protection of phagocytized bacteria against antibiotics. A new method for the evaluation of neutrophil granulocyte functions. Acta med. scand. *191* 383–387 1972.
14. *Solberg, C. O., Christie K. E., Larsen B. & Tonder O* Influence of antibodies and thermolabile serum factors on the bactericidal activity of human neutrophil granulocytes. Acta path. microbiol. scand. Sect. C, *84* 112–118, 1976.
15. *Solberg C. O & Hellum, K. B.* Influence of serum on the bactericidal activity of neutrophil granulocytes. Acta path. microbiol. scand. Sect. B, *81* 621–626, 1973.
16. *van Oss C J Woeppel, M S & Marquart S E* Immunoglobulins as aspecific opsonins. III. The opsonizing power of fragments of polyclonal and monoclonal immunoglobulin G. J Reticuloendothel. Soc. *13:* 221–230 1973

above for bacteria sensitized with intact IgG. There was no enhancement of either phagocytosis or intracellular killing of bacteria.

Influence of IgM

The influence on the phagocytic activity of the granulocytes of IgM at concentrations of 0.01–0.25 mg/ml was investigated. At a final IgM concentration of 0.25 mg/ml, the reduction in the number of colony forming units was approximately 60 per cent. However, controls showed that IgM alone, in the absence of granulocytes, reduced the number of colony forming units by as much as 50 per cent. Furthermore, the number of viable intracellular bacteria was even higher than that in the control, where immunoglobulins were substituted by HBSS. Accordingly, IgM did not stimulate the bactericidal activity but seemed to stimulate the phagocytic activity to a minor extent.

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DISCUSSION

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As the number of colony forming units [illegible] is a [illegible] parameter [illegible] agglutination of the bacteria may [illegible] result in a reduction in the number without killing the bacteria. However, the reduction in the number of colony forming units in a suspension of bacteria containing IgG was significantly higher in the presence of leucocytes than in the absence of leucocytes. In addition, the enhancing effect of IgG could easily be exhausted by absorption of the preparation with bacteria (14). This strongly suggests that the influence of IgG on the phagocytic and bactericidal activities of human neutrophils is due to absorption of the immunoglobulins on to the surface of the bacteria, followed by attachment of the sensitized bacteria to the cell membrane of the granulocytes (Fc receptors).

The pronounced killing of bacteria to be seen if procedure 2 was used and the relatively decreased phagocytosis at concentrations greater than 1 mg IgG/ml if procedure 1 was used, indicate that non-antistaphylococcal IgG may occupy the Fc receptors on the surface of the granulocytes. The results obtained by $F(ab')_2$ fragments which showed no difference in phagocytosis whether procedure 1 or 2 had been used are in keeping with this suggestion.

$F(ab')_2$ fragments had very little if any enhancing effect on the phagocytosis, and the reduction in the number of colony forming units was apparently due to agglutination of the bacteria. The bactericidal activity was not enhanced at all. This indicates that the Fc piece of the IgG molecule is important not only for the phagocytosis, but also for the intracellular killing of the bacteria. This is in agreement with the hypothesis that the attachment of sensitized bacteria to Fc receptors on the cell membrane of the granulocytes may also influence the bactericidal activity of the granulocytes, probably by acting as a stimulus by which the bactericidal enzymes may be released into the phagocytic vacuole (14, 15).

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REFERENCES

1 *Dossett J H., Kronvall G Williams, R. C.* J & Quie P G Antiphagocytic effect of staphylococcal protein A. J Immunol. *103* 1405–1410, 1969

2. *Fidalgo B V & Najjar V A.:* The physiological role of the lymphoid system. III. Leucophilic γ-globulin and the phagocytic activity of the polymorphonuclear leucocyte. Proc. Nat. Acad. Sci U.S.A. *57* 957–964 1967

3 *Fidalgo B. V & Najjar V A.:* The physiological role of the lymphoid system. VI. The stimulatory effect of leucophilic γ-globulin (leucokinin) on the phagocytic activity of human polymorphonuclear leucocyte. Biochemistry *6* 3386–3392, 1967

4 *Lenhart N A., Mudd S Yoshida, A. & Li I W* The common protein agglutinogen of *St phylococcus aureus*. I. Distribution in international serotypes and corresponding antibody in human populations. J Immunol. *91* 771–776 1963

5. *Li, I W & Mudd S* The heat-labile serum factor associated with intracellular killing of *St phylococcus aureus*. J Immunol. *94* 852–857 1965

6 *LoBuglio A. F Cotran, R S & Jandl J H.,* Red cells coated with immunoglobulin G Binding and sphering by mononuclear cells in man. Science *158* 1582–1585 1967

7 *Mietcher P A Spiegelberg, H & Benacerraf B.,* Studies on the mechanism of immune phagocytosis of sensitized bacteria and red cells by the reticuloendothelial system in mice. I : Halpern, B. N. (Ed.) Rôle du Système Réticulo-Endothélial dans l'Immunité Antibactérienne et Antitumorale CNRS, Paris, 1963

8. *Messner R. P & Jelinek J.,* Receptors for human γ-G globulin on human neutrophils. J Clin. Invest. *49* 2165–2171 1970.

9 *Nisonoff A Wissler F C & Lipman L. N.,* Properties of the major component of a peptic digest of rabbit antibody Science *132* 1770–1771 1960.

10. *Quie P G., Messner R P & Williams R. C.,* J Phagocytosis in subacute bacterial endocarditis. Localization of the primary opsonic site to Fc fragment. J Exp. Med. *128* 553–570 1968.

11 *Shayegani, M G., Hisatsune K & Mudd S.:* Cell wall component which affects the ability of serum to promote phagocytosis and killing of *St phylococcus aureus*. Infect. Immun. *2:* 750–756, 1970

12. *Solberg, C. O* Enhanced susceptibility to infection. A new method for the evaluation of neutrophil granulocyte functions. Acta path. microbiol. scand. Sect. B *80* 10–18, 1972.

13. *Solberg, C O.,* Protection of phagocytized bacteria against antibiotics. A new method for the evaluation of neutrophil granulocyte functions. Acta med. scand. *191* 383–387 1972.

14 *Solberg, C. O., Christie K. E., Larsen B. & T nder O.,* Influence of antibodies and thermolabile serum factors on the bactericidal activity of human neutrophil granulocytes. Acta path. microbiol. scand. Sect. C, *84* 112 118, 1976.

15 *Solberg, C O & Hellum, K. B.,* Influence of serum on the bactericidal activity of neutrophil granulocytes. Acta path. microbiol. scand. Sect. B *81* 621–626, 1973.

16. *van Oss C J., Woeppel, M S & Marquart S E.* Immunoglobulins as specific opsonins. III. The opsonizing power of fragments of polyclonal and monoclonal immunoglobulin G J Reticuloendothel. Soc. *13* 221–230 1973

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MACROPHAGE PROLIFERATION AND ACTIVATION DURING *TOXOPLASMA GONDII* INFECTION IN MICE RELATIONSHIP TO LYMPHOCYTE STIMULATION

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Reikvam A. Macrophage proliferation and activation during *Toxoplasma gondii* infection in mice Relationship to lymphocyte stimulation. Acta path. microbiol scand. Sect. C, 84 124–130 1976

Macrophage proliferation and activation as well as lymphocyte stimulation in the peritoneal cavities of mice were investigated during the course of a *Toxoplasma gondii* (Beverly strain) infection. Macrophage proliferation had started already after day one and reached a first peak on day 2 (^3H thymidine labelling index ~6 per cent) This proliferation was not accompanied by any notable lymphocyte stimulation An equally high L I for macrophages was found after injection of 0 9 per cent saline. From day 3–4 and peaking on day 7–11 a considerable blastoid transformation of lymphocytes occurred (maximum L I for lymphoid cells ~20 per cent on day 7) In parallel with this blastoid response a substantial macrophage proliferation took place (L.I ~8 per cent) Large numbers of activated macrophages also appeared during this period. DNA synthesizing cells were found even among the most highly activated macrophages. The results indicated that the early and the late macrophage proliferations were stimulated by different mechanisms.

Key words *Toxoplasma gondii* infection macrophage proliferation macrophage activation lymphocyte stimulation mice.

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The importance of macrophages for the resistance to infections and neoplasms has been clearly demonstrated (22) In the combat of intracellular micro-organisms, macrophages are particularly important since they are the ultimate killer cells in such infections. In order to perform this task the macrophages must however be activated (9 16 18, 26 27)

Toxoplasma gondii is an obligate intracellular protozoon which can be suppressed only if an adequate cellular immune response ensues (7 12 13) In a previous study it was shown that infection with this protozoon (Beverly strain) killed 15–30 per cent of the mice, deaths usually occurring between day 7 and 14 (26) The macrophages from mice surviving a Toxoplasma infection for 14 days were highly activated and had an increased

capacity to kill another Toxoplasma strain (RH strain) and a strain of *Listeria monocytogenes*. The immune apparatus is thus subjected to a most severe test in mice going through this type of infection. The present study was undertaken in order to examine macrophage proliferation and activation in the course of this critical infection. Another aim was to investigate the temporal relationship between the macrophage response and lymphocyte stimulation. This again might elucidate the process of macrophage proliferation, the controlling of which is poorly understood.

MATERIALS AND METHODS

Animals

Male NMRI/Bom mice, aged 1–4 months, weighing 20–30 gram, were used in all experiments.

Infection Procedure

Toxoplasma gondii strain Beverly was passed to new mice every third month. Brains of infected mice were dissected out, and homogenized with 0.9 per cent NaCl in mortar. The number of brain cysts per drop was counted and by dilution adjusted so as to give 5 cysts per 0.3 ml, which was the volume injected intraperitoneally to each new mouse.

Harvesting of Peritoneal Cells

At various times of Toxoplasma infection, the mice were killed and the peritoneal cells harvested. Cells destined for biochemical assays were harvested into medium 199 (Flow laboratories) containing 20 per cent foetal calf serum (Gibco) 50 I.U./ml of crystalline penicillin (Glaxo) and 50 µgram/ml of streptomycin (Glaxo). Cells to be used for evaluation of DNA-synthesis were harvested into the same type of medium containing in addition ^3H thymidine, 3 µCi/ml (Specific activity 2Ci/m-mol). Cells for biochemical assay were cultured on the bottom of Leighton tubes, while cells for autoradiography were cultured on 9 × 35 mm "flying cover slips" within the Leighton tubes. Further details have been given elsewhere (25, 26).

The cells were incubated at 37° C in an atmosphere of 5 per cent CO_2 in air. After 1½ hours, the cells for autoradiography were handled in the following manner. The Leighton tubes were agitated vigorously, the nonadherent cells were harvested and the adherent cells were washed twice with 2 ml medium 199. The non-adherent cells from each Leighton tube were centrifuged, a drop of bovine albumine was added to the cell sediment, and smears were made. These smears, as well as the cover slips containing the adherent cells, were processed for autoradiography.

The cells for biochemical assays were cultured for one hour. The non-adherent cells were then discarded and the adherent ones washed three times with 2 ml of 0.9 per cent NaCl. Subsequently the cells were wiped off the glass into 2 ml saline with a rubber policeman. These cell suspensions were stored at –20° C.

Autoradiography

Autoradiography was performed by the dipping technique using an Ilford L 4 size A" film. After exposure for 7 days, the films were developed and stained with Giemsa. Cells with 5 or more grains overlaying the nucleus were scored as labelled. In each coded smear 300 cells were scored. The percentages of labelled cells of the respective cell populations are expressed as the labelling index (L.I.).

Biochemical Assay

The cells were frozen and thawed 10 times on methanol dry ice bath in order to destroy the cells. *Acid phosphatase* (E.C. 3.1.3.2.) was assayed with β-glycerophosphate as substrate (2) using an incubation period of 4 hours. The liberated phosphate was quantitated by the method of *Fiske & Subbarow* (5). *Protein* was measured by the method of *Lowry et al.* using crystalline bovine serum albumin as a standard (15). More details have been given previously (26).

Cytochemistry and Autoradiography

In order to evaluate the acid phosphatase content of the individual DNA-synthesizing cells, the following double-labelling technique was performed. The cover slips containing macrophages were fixed in acetone and then stained, using the naphtol-AS-BI-phosphate method (3). The cells were exposed to the substrate for 20 minutes. After staining the cells were submitted to the ordinary procedure for autoradiography.

Colloidal Carbon

In order to identify phagocytic cells, the ability to take up colloidal carbon (C 11/1432 a, Gunther Wagner Pelikan, dilution 1:50) was examined.

RESULTS

All mice had signs of a severe infection during the second week. More than 50 per cent died and were not included in the present study.

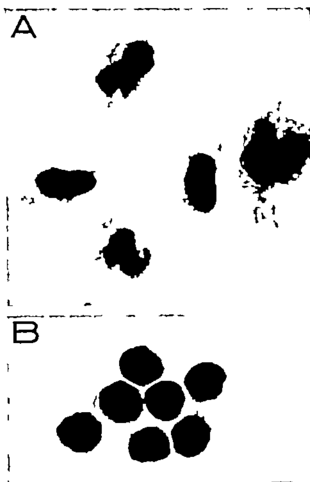


Fig 1 Peritoneal macrophages and lymphocytes 2 days after i p immunization with Toxoplasma cysts. A) Macrophages. B) Lymphocytes. Only small lymphocytes are seen.

Morphology of the Peritoneal Lymphocytes and Macrophages

The macrophages were identified by the functional test of their ability to adhere to glass, instead of relying merely on morphological criteria. In addition, the phagocytic ability of the adherent cells was examined in some experiments and virtually 100 per cent of them were heavily phagocytosing carbon.

The macrophages did not change in appearance during the first two days (Fig 1A) On day 3 many of the adhesive cells had a more monocyte-like appearance and less cytoplasm than the macrophages observed the preceding days. Later on, particularly on day 7 and 11 a more heterogeneous picture was found, but large macrophages, the appearance of activated cells, predominated (Fig. 2A)

The peritoneal lymphocytes had the appearance of small lymphocytes until days 3-4 (Fig 1B) Notable numbers of lymphoblasts then appeared and on day 7 and 11 a strong blastoid response was observed (Fig. 2C) the majority of the lymphocytes being blasts. Thereafter the blast response abated.

In addition granulocytes were seen, particularly during the first days. They were mainly present in the non-adherent cell population Only a minor proportion was glass-adherent after the vigorous shaking of the Leighton tubes.

Proliferation of Lymphocytes and Macrophages

The macrophage population showed a considerable proliferation already after one day



Fig 2 Peritoneal macrophages and lymphocytes 11 days after i.p. immunization with Toxoplasma cysts. A) Macrophages. Considerable variation in cell size. Note large cells. B) DNA-synthesizing macrophages. Macrophages differing markedly in cell size were synthesizing DNA. C) Lymphocytes. Note DNA synthesizing blasts.

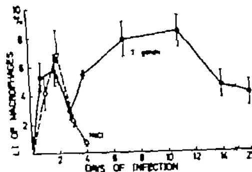


Fig 3 Labelling indices (L.I) of peritoneal macrophages after i p. immunization with *T gondii* Each mouse received approximately 5 brain cysts suspended in 0 9 per cent saline intraperitoneally The peritoneal macrophages were adhered to cover slips and thereby separated from the non-adherent cells by culturing for 1 ½ hours *in vitro* (^{3}H-thymidine containing medium) Control mice received 1 ml 0 9 per cent saline i p Means ± 1 SE are given At least 4 mice in each point. ●——● *T gondii* ○– –○ NaCl.

(Fig 3) On day 2, a further increase was found, whereafter a drop in the L.I was observed from day 2 to 3. The macrophage proliferation observed on the first two days was not accompanied by any obvious lymphocyte stimulation (Fig. 4)

From days 3–4 an increasing L.I was found in the lymphocyte population and also in the macrophage population (Fig 3 and 4) Even 3 weeks after the start of the infection, a considerable degree of proliferation was found in both cell populations.

In control mice injected with NaCl (sterile and pyrogen-free) a substantial macrophage proliferation was also found. Indeed the initial proliferation rate in this group was as high as in the Toxoplasma infected mice. However the proliferative response in the control mice fell back to negligible levels on day 4 This macrophage proliferation was not accompanied by any notable lymphocyte stimulation.

The tritium-labelled macrophages did not show any particular morphological characteristics as compared with the non-labelled macrophages in a given culture. Even among the largest, most typically activated macrophages, DNA-synthesizing cells were found (Fig. 2B)

Acid Phosphatase of Macrophages

Acid phosphatase content of macrophages, measured biochemically on a per mg protein basis, was markedly and significantly increased on day 11 (Table 1) A slight but not significant increase was found on day 4 and 21

Cytochemical staining for acid phosphatase, performed on day 11 showed that the macrophages stained rather strongly The staining intensity varied somewhat from cell to cell. Autoradiography performed on these smears did not reveal any particular pattern as to the acid phosphatase content of DNA synthesizing cells. Even cells which were most intensely stained were found to synthesize DNA.

DISCUSSION

The final aim of the immune response against the obligate intracellular *T gondii* is to provide macrophages capable of killing or at least restricting its intracellular growth. However the mice surviving the acute infection have not totally eliminated the protozoon. In these mice, the protozoon goes into

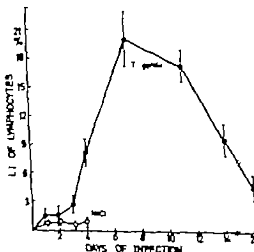


Fig 4 Labelling indices of lymphocytes in the same peritoneal cell populations as in Fig. 2

TABLE 1 *Acid Phosphatase Activity in Peritoneal Macrophages after Immunization with* Toxoplasma gondii

Days after Immunization	Enzyme activity (mU*/mg protein) Mean** ± SE
0 (non immun.)	2.2 ± 0.2
4	2.8 ± 0.6
11	11.3 ± 2.6
21	3.2 ± 0.7

* One unit of activity will hydrolyse one µmole of substrate in one minute.

** The enzyme activity in macrophages from 4–10 mice was determined on each of the days.

a cystic stage and remains in the body probably for life (8, *Reikvam* (unpublished results)). The present study shows that local macrophage proliferation is a prominent, and probably important, event in the response of the animals to this infection. It also shows that this macrophage proliferation as well as the lymphocyte stimulation are protracted responses in toxoplasma infected mice. The peak of the responses occurred during or slightly before the most critical post infection period when many of the mice succumbed. The time course of the macrophage proliferation observed after 3–4 days shows some resemblance to that reported to occur in mice infected with BCG (23) but is considerably more protracted. However, in that study (23) no distinction between peritoneal lymphocytes and macrophages was made and the BCG infection was established by the intravenous route.

Activated macrophages, as judged by morphology and by an increased content of acid phosphatase as well as by microbicidal capacity (26), were present in the second week of the infection. At this time a considerable macrophage proliferation also took place. Macrophage activation might be regarded as a cytoplasmic specialization designed to improve the microbicidal and cytotoxic capacity of the cells (10, 17). In many systems specialization is accompanied by a loss of proliferative capacity. In the present study the possibility must be considered that a non-proliferative activated macrophage population coexisted with a proliferative non-activated population. However, even among the largest and most typically activated macrophages, DNA synthesizing cells were found. Hence, proliferation seems to be a characteristic feature of the activated macrophages themselves.

How then is macrophage proliferation and activation initiated and regulated? Activation has been assumed to be due to soluble mediators (lymphokines) released from the lymphoblasts. *In vitro* experiments have confirmed this assumption and it has clearly been demonstrated that supernatants from stimulated lymphocytes can induce the typical features associated with activation, namely morphological changes (20, 21), increased content of lysosomes (20), and increased microbicidal capacity (9, 11, 27).

Stimulation of macrophage proliferation has also been suggested to be caused by lymphokines (19, 24), but this has been more difficult to demonstrate *in vitro* since macrophages are reluctant to divide when adhered to glass or plastic surfaces. However, *Godal et al.* (9) found an increase in the number of adherent macrophages after addition of lymphocyte supernatants and recently DNA synthesis was demonstrated in adherent macrophages after addition of inflammation exudate (1, 29). The exact nature of the mitogenic factor was not explored in the latter studies. The present experiments showed that the macrophage proliferation observed after about 3 days occurred in parallel with a strong lymphocyte stimulation, and thus might indicate a causal role for mediators from lymphoblasts.

The macrophage proliferation found during the first two days of Toxoplasma infection does not, however, fit in with this scheme. It was not accompanied by any notable lymphocyte stimulation and equally high proliferation rates could be obtained by the mere injection of saline. Such a rapid onset of proliferation in liver macrophages (Kupffer cells) has also been reported (4, 14, 28).

Moreover oestrogens, both natural and synthetic ones, are potent stimulators of the mononuclear phagocytes and can induce DNA synthesis in the liver macrophages after a lag phase of 16 hours (28, 14). *Forbes* reported proliferation of peritoneal macrophages of mice after repeated punctures of the peritoneal cavities (6). It is difficult to see that lymphokines should be operating under these circumstances. Furthermore lymphocyte transfer experiments, using *Listeria monocytogenes* as infecting agents, have demonstrated that lymphocytes from infected mice can confer resistance to normal mice acting mainly by stimulating macrophages. However such sensitized lymphocytes were not generated until 3 to 5 days after the start of the infection (16, 30). This time interval corresponds to that seen in the present study for onset of lymphocyte stimulation. Consequently it seems unlikely that in the present experiments a primary stimulation of lymphocytes resulting in the production of a mitogenic factor, the subsequent release of this factor and finally stimulation of macrophages to demonstrable DNA synthesis can have occurred within 24 hours.

In conclusion therefore, it seems pertinent to suggest that macrophage proliferation may be controlled in different ways. First, a short latency macrophage proliferation can be induced by appropriate, but yet not well-defined, stimulators. The primary target for these stimulators seems to be the monocyte/macrophage cell line. Second, macrophage proliferation can take place in the effector phase of a cellular immune response and, in this situation, presumably as a result of the influence of mediators from stimulated lymphocytes. During this type of proliferation activated macrophages appear and the activated cells themselves are capable of cell division.

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REFERENCES

1. *Adolphe M., Fontagne J., Pelletier M. & Giroud J. P.* Induction of DNA synthesis in rat macrophages in vitro by inflammatory exudate. Nature (Lond.) 253, 637. 1975.
2. *Appelmans F., Wattiaux R. & DeDuve C.* Tissue fractionating studies. 5. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. Biochem. J. 59, 438–445. 1955.
3. *Bancroft J. D.* An introduction to histochemical techniques. Butterworth, London 1967 pp. 192–194.
4. *Donald K. J. & Pound A. W.* Proliferation and migration of reticuloendothelial cells following infection of a tubercle bacillary lipid. Br. J. exp. Path. 54, 79–89. 1973.
5. *Fiske C. H. & Subbarow Y.* The colorimetric determination of phosphorus. J. biol. Chem. 66, 375–400, 1925.
6. *Forbes I. J.* Mitoses in mouse peritoneal macrophages. J. Immunol. 96, 734–743. 1966.
7. *Frenkel J. K.* Adoptive immunity to intracellular infection. J. Immunol. 98, 1309–1318, 1967.
8. *Frenkel, J. K.* Models for infectious diseases. Fed. Proc. 28, 179–190. 1969.
9. *Godal T., Rees R. J. W. & Lamvik J. O.* Lymphocyte-mediated modification of blood-derived macrophage function in vitro (inhibition of growth of intracellular mycobacteria with lymphokines. Clin. exp. Immunol. 8, 625–637. 1971.
10. *Hibbs J. B. jr, Lambert L. H. jr & Remington J. S.* Possible role of macrophage mediated nonspecific cytotoxicity in tumour resistance. Nature (Lond.) 235, 48–50. 1972.
11. *Jones T. & Youmans G. P.* The in vitro inhibition of growth of intracellular *Listeria monocytogenes* by lymphocyte products. Cell. Immunol. 9, 353–362, 1973.
12. *Jones, T. C.* Macrophages and intracellular parasitism. J. reticuloendoth. Soc. 15, 439–450. 1974.
13. *Jones T. C., Len L. & Hirsch J. G.* Assessment in vitro of immunity against *Toxoplasma gondii*. J. exp. Med. 141, 466–482, 1975.
14. *Kelly L. S., Dobson E. L., Finney C. R. & Hirsch, J. D.* Proliferation of the reticuloendothelial system in the liver. Amer. J. Physiol. 198, 1134–1138, 1960.
15. *Lowry O. H., Rosebrough N. J., Farr A. L. & Randall, R. J.* Protein measurement with the folin phenol reagent. J. biol. Chem. 193, 265–275. 1951.

Mackaness G B The influence of immunologically committed lymphoid cells on macrophage activity in vivo. J exp Med. *129* 973–992, 1969

Mackaness G B Cellular immunity In Furth R. van (Ed.) Mononuclear phagocytes. Blackwell Scientific Publications, Oxford 1970 pp 461–476

McGregor D D & Koster F T The mediator of cellular immunity IV Cooperation between lymphocytes and mononuclear phagocytes. Cell Immunol. *2* 317–325 1971

More D G Penrose J M., Kearney R & Nelson D S Immunological induction of DNA synthesis in mouse peritoneal macrophages. Int. Arch. Allergy *44* 611–630 1973

Nath I Poulter L. W & Turk J L Effect of lymphocyte mediators on macrophages in vitro A correlation of morphological and cytochemical changes. Clin. exp Immunol. *13* 455–466 1973

Nathan C F Karnovsky M L. & David J R. Alterations of macrophage functions by mediators from lymphocytes. J exp. Med. *133* 1356–1376, 1971

Nelson D S Immunity to infection allograft immunity and tumour immunity parallels and contrasts. Transplant. Rev *19* 226–254 1974

North R J Cellular kinetics associated with the development of acquired cellular resistance. J exp Med. *130* 299–314 1969

24 *North R J* The mitotic potential of fixed phagocytes in the liver as revealed during the development of cellular immunity J exp. Med. *130* 315–326 1969

25 *Reikvam A & Høiby E. A.* Phagocytosis and microbicidal capacity of mouse macrophages non-specifically activated in vitro. Acta path. microbiol. scand. Sect. C, *83* 121 128, 1975

26 *Reikvam A Grammeltvedt R. & Høiby E A* Activated mouse macrophages morphology lysosomal biochemistry and microbicidal properties of in vivo and in vitro activated cells. Acta path. microbiol. scand. Sect. C, *83* 129–138, 1975

27 *Simon H B. & Sheagren J N* Cellular immunity in vitro. I Immunologically mediated enhancement of macrophage bactericidal capacity J exp Med *133* 1377–1389 1971.

28. *Warr G W & Sljivic V S* Origin and division of liver macrophages during stimulation of the mononuclear phagocyte system. Cell Tissue Kinet. *7* 559–565 1974

29 *Wynne K M Spector W G & Willoughby D A* Macrophage proliferation in vitro induced by exudates. Nature (Lond.) *253* 636–637 1975

30. *Zinkernagel R. M Blanden, R V & Langman R E.* Early appearance of sensitized lymphocytes in mouse infected with *Listeria monocytogenes* J Immunol. *112* 496–501 1974

Acta path. microbiol. scand. Sect. C, 84: 131–134, 1976

INFLUENCE OF HYDROCORTISONE ON UPTAKE AND ELIMINATION OF ³²P LABELLED *E. COLI* BY RAT POLYMORPHONUCLEAR NEUTROPHILS (PMN) *IN VITRO*

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Baardsen, A. Influence of hydrocortisone on uptake and elimination of ³²P-labelled *E. coli* by rat polymorphonuclear neutrophils (PMN) *in vitro*. Acta path. microbiol. scand. Sect. C, 84: 131–134, 1976.

Hydrocortisone was tested for inhibition of uptake and of elimination of *E. coli* by monolayers of rat peritoneal PMN. ³²P was used as label of the bacteria and their degradation products eliminated from the phagocytes. The cellular uptake was reduced by hydrocortisone (1–2 mg per ml) both in the presence and absence of serum, while the elimination of bacterial label was reduced by 0.5 mg per ml and higher concentrations of the drug.

Key words: Hydrocortisone; neutrophils; phagocytosis; opsonins; *E. coli*.

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The influence of glucocorticosteroids (steroids) on the intracellular killing of bacteria by polymorphonuclear neutrophils (PMN) has not been definitely settled (3, 5, 14, 17). It may be difficult to distinguish between interference with the degradation of phagocytized bacteria and interference with the cellular uptake (4, 7, 18).

Steroids impair some of the biochemical events within the phagocytes triggered by ingestion of bacteria (7, 9, 15). The importance of such observations to the rate of intracellular killing, however, has been difficult to establish. Pertinent investigations based on conventional microbiological methods and viable counts have led to conflicting results (3, 9, 11, 13, 17). A number of uncertain factors are involved in these methods (7, 10, 16) which are limited to a low ratio of bacteria per phagocyte.

This paper describes a different approach to this problem. The influence of hydrocortisone (HC) on the bactericidal activity of rat PMN has been evaluated by assay of labelled bacterial breakdown products eliminated from the phagocytes in the postingestive period. The assay system has previously been described (10).

In another study (2), the interest was focused on the influence of HC on PMN phagocytosis and interference with serum opso-

nins. As a part of the present study the direct influence of HC on PMN uptake of *E coli* has been evaluated in the absence of serum

MATERIALS AND METHODS

The present assay system utilizes monolayers of glass-adherent rat peritoneal PMN incubated in tissue culture tubes with ^{32}P-labelled *E coli* as previously described (10 16) At the termination of the standard period of incubation of 15 min at 37 C, the monolayers were washed 4 times in a Krebs-Ringer phosphate buffer with 10 mM glucose (KRG) which was also used as the basic medium Fresh (frozen) rat serum (2) was added to a concentration 1 10 as stated in legends to Figs. 2–3 Hydrocortisone succinate (Solu Cortef®) The Upjohn Company Kalamazoo Mich., U S.A.) was added to the medium in final concentrations 0 25–2 mg per ml as stated in legends to Figs. 1–3

The elimination (E_t) of bacterial label from PMN to the extracellular medium after a certain postingestive period of time t (0–180 min) was expressed as percentage of the calculated total uptake (10)

The two-samples ranks test of Wilcoxon White was used for statistical analysis (8)

RESULTS

I Influence of HC on the Uptake of E coli by PMN in the Absence of Serum

The cellular uptake of *E coli* was significantly ($p<0.01$) reduced by HC at the concentration 2 mg per ml. The results of two experiments are shown in Fig 1

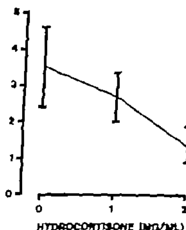


Fig 1 Influence of hydrocortisone on uptake of ^{32}P labelled *E. coli* by PMN in the absence of serum. I = 1 standard deviation.

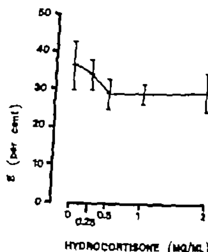


Fig 2 Influence of hydrocortisone on elimination (E) of bacterial label from PMN 180 min after termination of uptake of ^{32}P-labelled *E. coli*. E is expressed as the percentage radioactivity expelled to the extracellular medium in relation to total uptake The drug was added to the medium simultaneously with bacteria and serum, and not to the postingestive medium. I = 1 standard deviation.

II Influence of HC on the Elimination (E) of Bacterial Label from PMN

A In the presence of HC during ingestion In the absence of serum the uptake of radioactivity in the presence of 2 mg HC per ml (Fig 1) was too low for accurate determination of E.

In the presence of serum HC reduced the cellular uptake of bacteria, but not below that of KRG controls (in the absence of serum and HC) The total uptake of radioactivity was therefore sufficiently high for determination of E by means of the present method. The results of three experiments are shown in Fig 2 and it follows that E was reduced in the presence of 0.5–2 mg HC per ml ($p<0.01$/HC 0.5 mg/ml)

B In the presence of HC in the postingestive medium In the absence of serum and the presence of 2 mg HC per ml in the post ingestive medium, E was found to be lower than that to be found in the absence of HC. (At 120 min of the postingestive period this reduction was approximately 10 per cent,

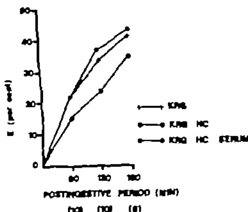


Fig 3 Elimination (E) of bacterial label from PMN after constant uptake of ^{32}P-labelled *E.coli*. (E explained in legend to Fig 2) Influence of hydrocortisone (HC) 2 mg per ml and serum (10 per cent) in the postingestive medium Number of observations is indicated in brackets

p<0 01) In the presence of serum, however the same concentration of HC did not affect E (Fig 3

DISCUSSION

The demonstrated HC-induced inhibition of the cellular uptake in the absence of serum may indicate a direct effect of the steroid on the phagocyte and/or bacteria. This effect could not be demonstrated by pre-incubation of PMN or *E. coli* with the steroid (2) Thus, a rapid reversibility of the steroid-induced interference with PMN-functions is indicated In the present system, the cellular uptake is inhibited by HC in the presence of serum opsonins as well (2) Comparison of these previously described data with the present results suggests either that HC makes the phagocyte and/or bacteria insensitive to the actions of opsonins or the latter are blocked at the cellular level and not in the fluid phase (6)

The present parameter E may depend upon 1) the rate of intracellular degradation of the bacteria ingested and 2) the rate by which the degradation products are transported out of the phagocyte. Theoretically HC might interfere with both factors.

After uptake of bacteria under standard conditions, the presence of HC in the post ingestive medium reduced E when serum was absent. This HC-induced effect disappeared, however when serum was added to the medium, possibly due to binding of free hormone to serum factors (12) Hence it appears that the concentrations of free hormone needed to interfere with the transport mechanism are considerably higher than those needed to impair the cellular uptake of *E coli* by PMN (2)

Recently *Ackerman & Beebe* (1) found that dexamethasone reduced lysosomal enzyme release by inhibiting cellular uptake (of zymosan particles) in guinea pig peritoneal leucocytes. Similarly *Wiener et al* (18) observed in cultured mouse peritoneal macrophages that HC lowered the uptake of isotopically labelled *Salmonella typhimurium* but lacked any effect on their degradation.

In the present study HC reduced E if the hormone was incubated with PMN in the uptake phase. This means that the bacterial constituents are eliminated from PMN by HC at a rate reduced proportionally more than the uptake is reduced. However this effect was not found if HC concentrations below 0.5 mg per ml were used. As previously discussed (10) this concentration is not reached in patients. Considered on the basis of the present results is seems therefore unlikely that HC should impair the uptake and elimination of *E. coli* by PMN to any measureable extent *in vivo*

REFERENCES

1 *Ackerman N R. & Beebe J R.*, Effects of pharmacologic agents on release of lysosomal enzymes from alveolar mononuclear cells (and peritoneal leucocytes) J Pharmacol Exp. Ther *193* 603–613 1975
2 *Bourdon A., Tripptad A & Midtvedt T.*, In vitro influence of glucocorticosteroids on opsonic and bactericidal activities against *Escherichia coli* in rat serum. Acta path. microbiol. scand. Sect. B *82* 696–700 1974
3 *Dale D C & Petersdorf R. G* Corticosteroids and infectious diseases. Med. Clin. North. Am. *57*: 1277–1287 1973.
4. *Elsbach P.*: On the interaction between phag

ocytes and microorganisms. N Engl. J Med *298* 846–852 1973

5 *van Furth R & van Zwet T L.* In vitro determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes. In Weir D M (Ed): *Handbook of Experimental Immunology* sec. Ed Vol 2, Blackwell Sc. Publ Oxford/London/Edinburgh 1973 p 36 1–36 23

6 *Gewurz H Wernick P R Quie P G & Good R A* Effects of hydrocortisone succinate on complement system Nature *208* 755–757 1965

7 *Karnovsky M L.* Biochemical aspects of the functions of polymorphonuclear and mononuclear leucocytes. In Bellanti, J A & Dayton D H. (Ed.) The phagocytic cell in host resistance Raven Press, New York 1975 p. 25–43

8 *Mainland D* Elementary medical statistics. 2 ed. W B. Saunders Company Philadelphia and London 1964

9 *Mandell G L Rubin W & Hook E W* The effect of an *NADH* oxidase inhibitor (hydrocortisone) on polymorphonuclear leucocyte bactericidal activity J Clin. Invest. *49* 1381–1388 1970

10. *Midtvedt T & Baardsen A* Elimination of ingested ^{32}P labelled *E. coli* from rat polymorphonuclear neutrophils (PMN) Evaluation of a method Acta path. microbiol. scand. Sect. C, *84* 100–104 1976.

11 *Renner E. D Webel, M L. & Rits, Jr R E* The effect of methylprednisolone on leucocyte function. RES *14* 530–537 1973.

12 *Rosner W* Recent studies on the binding of cortisol in serum. Journal of Steroid Biochemistry (J.S.B.) *3* 531–542 1972.

13 *Ruutu T & Kosunen T U.* *In vitro* effect of anti inflammatory agents on phagocytosis and bacterial killing by human neutrophilic leucocytes. *Acta pharmacol. et toxicol. 31* 226–237 1972

14 *Sneiderman C A & Wilson J W* Effects of corticosteroids on complement and the neutrophilic polymorphonuclear leucocyte. Transplant. Proc. *7* 41–48 1975

15 *Spirer Z Zakuth I Weisman Y & Bogair N* The effect of corticosteroids on the bactericidal activity of leucocytes. Experientia *30* 303–304 1974

16 *Trippestad A & Midtvedt T* Phagocytosis of ^{32}P-labelled *E. coli* by rat peritoneal polymorphonuclear leucocytes. Evaluation of a method Acta path. microbiol. scand. *74* 259–273 1968.

17 *Zurier R B & Weissmann G.* Anti-immunologic and anti-inflammatory effects of steroid therapy Med. Clin. North. Am. *57* 1295–1307 1973

18 *Wiener E., Marmary Y & Curelaru Z.* The *in vitro* effect of hydrocortisone on the uptake and intracellular digestion of particulate matter by macrophages in culture Lab. Invest. *26* 220–226 1972.

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STUDIES ON THE *TREPONEMA PALLIDUM* IMMOBILIZING ACTIVITY IN NORMAL HUMAN SERUM

2 *Serum Factors Participating in the Normal Serum Immobilization Reaction*

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Hederstedt, B. Studies on the T *ponema pallidum* immobilizing activity in normal human serum. 2. Serum factors participating in the normal serum immobilization reaction. Acta path. microbiol. scand. Sect. C, *84* 135–141 1976

The T *pallidum* immobilization reaction to be achieved by unheated normal serum was found to be complement dependent and the results presented suggested that complement was activated in the classical pathway Besides complement, an immobilizing antibody of the IgM class was necessary for the immobilization reaction to occur Lysozyme exerted an enhancing effect on the normal serum immobilization reaction.

Key words *T ponema pallidum* immobilizing activity normal human serum.

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In a previous paper (*Hederstedt* 1974) a method for an *in vitro* determination of the *T pallidum* immobilizing activity of human normal sera (NS) has been described. As compared with the conventional TPI test, this method involves a simplified treponemal medium and a shorter incubation period (two hours instead of 18)

In the present study attempts were made to identify the serum factors involved in the immobilizing action of human NS upon *T pallidum*

MATERIALS AND METHODS

Normal ser (NS) obtained from healthy persons were stored in 2 ml aliquots at –60 C until used.

Paired ser (rom mother and umbilical ord On the day of delivery blood samples were taken from healthy mothers and their full-term newborns. The sera obtained were stored in the same way as the NS.

Heat treatment of the sera was performed in the following manner· Sera were thawed at room temperature and distributed in 2 ml aliquots into preheated Erlenmeyer flasks with a bottom diameter of 8 cm. The flasks were then placed in water baths at different temperatures for varying periods of time. Under such conditions, the temperature equilibrium was reached within seven minutes.

T *pallidum immobilizing activity of normal sera* was determined by a method described previously (*Hederstedt* 1974)

Haemolytic complement activity of the sera on sensitized sheep erythrocytes was determined by the method of Mayer (*Kabat & Mayer* 1961) slightly modified the total olume of the lytic system was 1.25 ml containing 0.75 ml of serum dilution and 0.5 ml of a suspension of sensitized sheep red cells. The serum titres were expressed as the reciprocal of the serum dilutions that gave 50 per cent haemolysis (CH_{50}) The standard deviation of the results obtained by this method in duplicate tests and in tests carried out on various days was found to be

+ 0.006 and + 0.027 log units, respectively, (*Hederstedt* 1961)

Chelator Ethyleneglycoltetra-acetic acid (EGTA E. Merck AG 61 Darmstadt) was prepared in a 0.1 M stock solution 0.05 ml of which was added to 1 ml serum to obtain a final concentration of approximately 0.005 M

Absorption of the sera with T. pallidum A suspension of freshly isolated treponemes (virulent *Nichols strain*) was purified by high speed centrifugation in preformed potassium tartrate density gradients (*Rathlev* & *Pfau* 1965) After dialysis against saline two parts of the treponemes were added to seven parts of the serum to be absorbed. The absorption was carried out at 0 C for 30 minutes.

Absorption of the sera with anti IgM and IgG IgM was removed from the sera by affinity chromatography using the IgG fraction of a goat anti human IgM serum (Dakopat, Copenhagen, Denmark) coupled to CNBr-activated Sepharose 4B (*Pharmacia, Uppsala, Sweden*) Prior to the immuno-absorption, the sera were dialysed against buffered 0.01 M ethylenediaminetetra-acetic acid (EDTA) and the immuno-absorption was performed at 4 C, also in the presence of 0.01 M EDTA The effluent from the Sepharose column was concentrated to the original serum volume against polyethyleneglycol at 4 C and EDTA was removed by dialysis against 0.01 M phosphate-buffered saline pH 7.4 By way of comparison the sera were absorbed as above with a goat anti human IgG serum (Dakopat)

The absorbed sera were tested for presence of IgM and IgG by radial immunodiffusion (*Mancini et al.* 1965) Haemolytic complement activity was tested prior to and after the absorption.

Preparation of C1q C1q was purified from human NS as described by *Agnello et al.* (1970)

Treatment of the sera with 2 mercapto-ethanol was carried out according to the method of *Schrohenloher et al.* (1964)

Lysozyme Hen egg-white lysozyme (Worthington Biochem. Corpor Freehold N J) and human urine lysozyme (*Laboratory Equipment Displays, Los Angeles, California*) were used The lysozyme activity was measured, using the lysoplate method of *Lundblad et al.* (1974) By this method, the presence of lysozyme could be measured even in concentrations as low as 0.05 µg/ml. The removal of lysozyme from the sera was effected by adsorption onto bentonite (*Wardlaw* 1962)

RESULTS

1 *Participation of Complement*

It was found in a previous study (*Hederstedt* 1974) that human NS lost their lytic activity against sheep red cells and immobilizing activity against *T. pallidum* upon heating at 56° C for 30 minutes. In the present investigation, human NS were tested after heating/storage at different temperatures for varying periods of time.

Heating at 48–49 C of sera from five healthy blood donors abolished the immobilizing as well as haemolytic activities of these sera within one hour The titres of these two activities of one of the sera are recorded in Table 1 Storage of human NS at various temperatures (from +35° to −60 C) revealed that these two activities of serum were not significantly reduced at −60 C even after 16 months. At room temperature (+20° C) a 50 per cent reduction of the two activities were noticed after 5–10 days (Table 2)

TABLE 1 *Effect of Heating at Various Temperatures for One Hour upon Immobilizing and Haemolytic Complement Titres of a Normal Human Serum*

Temperature	Immobilizing titre (IT_{50})	Complement haemolytic titre (CH_{50})
Unheated	9.0*	139§
+46 C	7.4	119
+47 C	4.1	85
+48 C	<2.0	29
+49 C	<2.0	<5

* Reciprocal of serum dilution that gave 50 per cent immobilization.

§ Number of 50 per cent haemolytic units/ml.

TABLE 2 *Effect of Length of Storage at Various Temperatures upon Immobilizing and Haemolytic Complement Activities of Normal Human Sera*

Temperature	Average storage period causing a 50 per cent reduction in	
	Immobilizing titre (IT_{50})	Complement haemolytic titre (CH_{50})
+35 C	3 days*	5 days
+20 C	5 days	10 days
+ 4 C	14 days	18 days
60 C	>16 months	>16 months

* Mean values obtained by testing four sera.

Chelating serum with ethyleneglycoltetra-acetic acid (EGTA) permits alternate but

inhibits classic complement pathway activation (*Fine* 1974) Addition of EGTA to a final concentration of 0.005 M in serum totally inhibited the *T pallidum* immobilizing activity of NS. If Ca^{++} but not Mg^{++} were added to the EGTA treated serum this activity would be restored (Table 3)

TABLE 3 *Effect of Adding Mg^{++} and C^{++} to the Imbobilizing Capacity of EGTA Treated NS*

EGTA	Mg^{++}	Ca^{++}	Per cent immobilization
0 005	–	–	0
0 005	0.01	–	4
0 005	0.04	–	0
0.005	–	0.01	96
0 005	–	0.02	100

Molarity added to serum.

The immobilizing titres (IT_{50}) and the haemolytic complement titres (CH_{50}) of sera from 30 healthy persons were found to vary between <2.0–9 4 and 119–263, respectively There existed no direct correlation between these two serum activities, the correlation coefficient being –0 11

2. *Participation of an Immobilizing Serum Factor*

Absorption at 0 C with 6.5 × 10^7 treponemes removed the immobilizing activity from serum but did not appreciably affect its total haemolytic activity (Table 4)

TABLE 4 *Effect of Absorption with* T pallidum *on Immobilizing and Complement Haemolytic Activities of Normal Human Ser*

Normal human serum	Immobilizing titre (IT_{50})	Complement haemolytic titre (CH_{50})
Unabsorbed	12.0	168
Absorbed with *T pallidum*		
1 6 × 10^7	12 1	138
3 2 × 10^7	8.2	154
6.5 × 10^7	<2 0	148
13.0 × 10^7	<2.0	150

Removal of IgM from NS by a single absorption with goat antihuman IgM serum abolished the immobilizing activity of NS (Table 5) and reduced its haemolytic complement activity from 146 to 48 CH_{50} units. The addition of 30 µg of purified C1q per ml of the absorbed NS increased its haemolytic complement activity from 48 to 64 CH_{50} units, but did not restore its capacity of immobilizing *T pallidum*. Neither was its immobilizing activity restored by the addition of 40 µg of lysosome per ml of serum. Following a single absorption with anti-IgG NS did not lose more than about 10 per cent of its IgG A reduction of its haemolytic complement activity from 146 to 35 CH_{50} units was, however recorded, and yet almost 50 per cent of its immobilizing activity remained.

TABLE 5 *Effect of the Absorption with Rabbit Anti-Human IgM and IgG on the Immobilizing Activity of Normal Human Serum*

Normal human serum	Immobilizing titre (IT_{50})	Complement haemolytic titre (CH_{50})
Unabsorbed	12 8	146
Absorbed with anti-IgM	<2.0	48
Absorbed with anti-IgM; purified C1q added	<2.0	64
Absorbed with anti-IgG	5 4	35

The treatment of five human NS with 2-mercapto-ethanol resulted in abolishing their immobilizing capacity and also the major part of their haemolytic activity The addition of complement (parent serum, deprived of immobilizing activity by absorption at 0 C with *T pallidum*) did not restore their immobilizing activity

Sera from 19 mothers and from the umbilical cords of their 19 newborns were examined. Immobilizing activity was demonstrated in almost all the mothers sera and only in two of the cord sera (Table 6) The haemolytic C titres were higher in the material (423–172

TABLE 6 *Immobilizing Activity in 19 Maternal and Umbilical Cord Sera*

	Number of sera showing immobilizing titre (IT_{50})							
	<2 0	2 0	2 8	4.0	5 6	8 0	11.2	Total reactive
Maternal sera	1	4	4	5	3	1	1	18
Cord sera	17	0	1	1	0	0	0	2

TABLE 7 *Effect of Bentonite Absorption on Lysozyme Content Immobilizing Activity and Haemolytic Complement Activity of Four Normal Human Sera*

Serum No	Lysozyme conc. (µg/ml)*		Immobilizing titre (IT_{50})		Compl. haemol. titre (CH_{50})	
	before absorption	after absorption	before absorption	after absorption	before absorption	after absorption
1	5.3	<0 05	2.9	2 0	164	154
2	4 4	<0 05	14.2	9 6	170	164
3	3 9	<0 05	6 9	4.2	208	200
4	3 4	<0 05	14.8	9 6	154	160

* The lysozyme concentration of the treponemal suspension was found to be 0.2 µg/ml.

mean 254) than in the umbilical cord sera (200–44 mean 116)

3 *Participation of Lysozyme*

The participation of serum lysozyme in the immobilization reaction was examined in a system in which serum was almost completely depleted of lysozyme and in systems containing varying amounts of added lysozyme

The removal of lysozyme from the sera was carried out by absorption of the sera with bentonite, involving little or no loss of complement haemolytic activity The immobilization titres of such absorbed sera were markedly reduced (Table 7)

The lysozyme content of a human NS was reduced from 3 7 µg to 0 05 µg upon bentonite absorption while its immobilizing titre was reduced from 32 7 to 9 1 It appears from Fig 1 that the addition of increasing amounts of the lysozyme to this absorbed serum brought about increasing immobilization titres. Thus, 4 µg of lysozyme added to the serum restored its immobilizing activity while 10–20 µg of lysozyme caused a doubling of the immobilizing titre. This finding indicated that serum lysozyme participates in treponema immobilization caused by human NS, although no correlation between lysozyme content of individual sera and their immobilizing titre was found (Table 7)

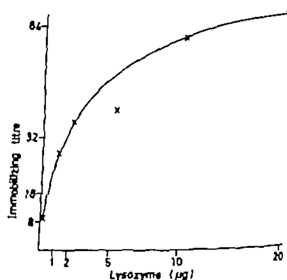


Fig 1 Immobilizing titres, plotted on an arithmetic scale, of one normal human serum at various concentrations of human lysozyme in the reaction mixture

DISCUSSION

In the present study of the *T pallidum* immobilization reaction by NS heating at 48–49 C for 30 minutes abolished or reduced

the haemolytic as well as the immobilizing capacity of NS. These findings suggested that the *T. pallidum* immobilizing activity in NS was complement dependent, just as in immune serum (*Nelson & Mayer* 1949).

In a previous study of the optimal conditions of the NS immobilization reaction (*Hederstedt* 1974) EDTA was found to inhibit the reaction. An enhancing effect of Mg^{++} on the reaction was demonstrated which would suggest that complement was activated through the alternate, properdin, pathway first described by *Pillemer et al.* (1954). However in the present study ethyleneglycoltetra-acetic acid (EGTA) was found to inhibit the immobilizing activity of NS. EGTA chelates in serum Ca^{++} much more efficiently than Mg^{++} and in EGTA treated serum, complement can be activated via the alternate but not the classical pathway (*Fine et al.* 1972, *Fine* 1974). The addition of Ca^{++} but not Mg^{++} to the EGTA treated NS restored its immobilizing capacity indicating that in the immobilization reaction by NS complement activates via the classical pathway.

Absorption of NS with highly purified treponemes at 0°C abolished the immobilizing activity of NS reducing only slightly its haemolytic activity. This finding might suggest that an immobilizing antibody was absorbed at the absorption. Removal of IgM from NS by immuno-absorption resulted in a total loss of its immobilizing capacity. At the absorption, a concommittant reduction by more than 60 per cent of the haemolytic complement activity of NS was recorded, which could explain the loss of the immobilizing activity. C1q is known to react with immune complex in the presence of EDTA (*Muller Eberhard & Kunkel* 1961, *Agnello et al.* 1970). The addition to the absorbed serum of purified C1q restored part of its haemolytic complement activity but not its immobilizing activity. Moreover the amount of IgG that was removed upon absorption of NS by an anti-IgG preparation was equal to the amount of IgM that was absorbed in the first experiment. Hereby the haemolytic complement activity of the absorbed NS was reduced to around 70 per cent, while around 50 per cent of the immobilizing capacity remained. This decrease of the immobilizing activity was apparently due to the loss of haemolytic complement. The present findings might indicate that the total loss of NS immobilizing activity noticed in the first immunoabsorption experiment was due to the removal of IgM and not to the reduced concentration of complement.

That the antibody involved in the immobilization reaction would be of the IgM class was also suggested by the finding that it was sensitive to 2-mercaptoethanol treatment.

In 17 out of 19 umbilical cord sera tested, no immobilizing activity could be demonstrated. The total haemolytic complement activity of these sera was found to be sufficiently high for *T. pallidum* immobilization. This finding suggested that, in the majority of cases, the immobilizing serum factor did not pass the placenta. This is also consistent with the suggestion that the NS immobilizing antibody was of the IgM variety. Intra-uterine production of IgM by the foetus is known to occur and might be the reason why immobilizing activity was demonstrated in two of the cord sera.

Immobilisins have been demonstrated in more than 80 per cent of human NS (to be published). NS antibodies of various classes against *T. pallidum* were demonstrated by *Julian et al.* (1970) who used an indirect fluorescent antibody (FA) technique. IgG in 100, IgM in 58 and IgA in 20 per cent of the sera tested. Upon absorption with a Reiter sorbent, a procedure known to abolish the FTA activity of NS (*Hunter et al.* 1964) no significant influence on the immobilizing activity was demonstrated. Moreover in NS freed from immobilizing activity by absorption with a suspension of *T. pallidum*, the FTA activity did not appear to be influenced. These unpublished results suggested that the specificity of the immobilisins of NS was not the same as that of the fluorescence-detected antibodies.

Recently *Reed & Albright* (1974) showed that "bacteriolysis required small amounts of

antibody but as little as 0.02 mg of a 19S fraction from normal serum restored full killing capacity to 1 ml of antibody depleted serum. Analogous to this finding the concentration of the T. pallidum immobilizing IgM antibodies of NS might be too low to be detected by the indirect FA technique.

Lysozyme is known to exert its effect after the specific interaction between antibody and antigen. Lysozyme is normally present in human sera in concentrations around 2–7 micrograms per ml (*Lundblad et al.* 1974). After NS had been depleted of lysozyme by bentonite absorption, its immobilizing activity was significantly reduced (Fig. 1). It could be restored and even enhanced if hen egg white lysozyme (or human serum lysozyme—unpublished result) were added to the absorbed sera, indicating that lysozyme took part in the reaction.

A similar effect of lysozyme on the immobilization reaction of syphilitic serum was reported by *Metzger et al.* (1961). It was suggested by *Metzger* (1962) and *Muller et al.* (1973) that the immune serum immobilization reaction was even lysozyme-dependent.

Treponemal suspensions derived from rabbit testicles contain various amounts of lysozyme (*Metzger* 1963). In the reaction mixtures used in the present experiments minute amounts of rabbit lysozyme might influence the immobilization reaction of NS. Thus the present results have only indicated that lysozyme exerts an enhancing effect on the NS immobilization reaction, not that it is necessary for the reaction to occur.

A suggestion that the NS immobilization reaction is not lysozyme-dependent would be in agreement with the findings obtained by *Adinolfi et al.* (1966). Using a bacteriolytic system they observed that IgA antibodies lysed bacteria only in the presence of both complement and lysozyme, while IgM antibodies were bacteriolytic in the presence of complement alone, without lysozyme.

In conclusion the results presented indicate that the NS immobilization reaction is dependent on complement and an immobilizing IgM antibody and that lysozyme enhances the reaction.

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REFERENCES

Adinolfi M., Glynn A. A., Lindsay M. & Milne C. M.: Serological properties of γA antibodies to *Escherichia coli* present in human colostrum. Immunology *10*, 517–526, 1966.

Agnello V., Winchester R. J. & Kunkel H. G.: Precipitin reactions of the C1q component of complement with aggregated γ-globulin and immune complexes in gel diffusion. Immunology *19*, 909–919, 1970.

Fine D. P.: Activation of the classic and alternate complement pathways by endotoxin. J. Immunol. *112*, 763–769, 1974.

Fine D. P., Marney Jr. S. R., Colley D. G., Sergent J. S. & Des Prez R. M.: C3 shunt activation in human serum chelated with EGTA. J. Immunol. *109*, 807–809, 1972.

Hederstedt B.: Studies on the complement activity in the *Treponema pallidum* immobilization (TPI) test. A comparative study of the immobilizing and haemolytic complement activity. Acta path. microbiol. scand. *53*, 180–190, 1961.

Hederstedt B.: Studies on the *Treponema pallidum* immobilizing activity in normal human serum. I. A method. Acta path. microbiol. scand. Sect. B *82*, 185–200, 1974.

Hunter E., Deacon W. E. & Meyer P. E.: An improved FTA test for syphilis, the absorption procedure (FTA ABS). Publ. Hlth Rep. *79*, 410–412, 1964.

Julian A. J., Logan L. C., Norins L. C. & Cohen J. R.: Immunoglobulin class of natural immune antibodies reactive with *Treponema pallidum*. Infection and Immunity *1*, 555–558, 1970.

Kabat E. A. & Mayer M. M.: Exp. Immunochemistry. Charles C. Thomas, Springfield, Ill. Second edition, 1961.

Lundblad G., Hederstedt B., Lind J. & Steby M.: Chitinase in goat serum. Preliminary purification and characterization. Eur. J. Biochem. *46*, 367–376, 1974.

Mancini G., Carbonara A. O. & Heremans J. F.: Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry *2*, 235–254, 1965.

Metzger M., Hardy J., P. H. & Nell, E. E.: Influence of lysozyme upon the treponeme immobilization reaction. Amer. J. Hyg. *73*, 236–244, 1961.

Metzger M.: A study of the role of serum and

tissue lysozyme upon the treponeme immobilization reaction. Amer J Hyg *76* 267 275 1962.
Metzger M Lysozyme activity of rabbit sera and testicular tissue fluid during the development of syphilitic lesions. Arch. Immunol. Ther exp. *11* 608, 1963
Muller F Feddersen H & Segerling, M Studies on the action of lysozyme in immune immobilization of *Treponema pallidum* (Nichol Strain) Immunology *24* 711–719 1973
Muller Eberhard H J & Kunkel, H G Isolation of thermolabile serum protein which precipitates γ-globulin aggregates and participates in immune hemolysis. Proc. Soc. exp. Biol. Med. *106* 291 295, 1961.
Nelson, J R. A. & Mayer M M : Immobilization of *Treponema pallidum* in itro by antibody produced in syphilitic infection. J exp. Med *89* 369–393 1949
Pillemer L Blum L Lepow I H., Ross O A., Todd E. W & Wardlaw A C The properdin system and immunity I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. Science *120* 279–285 1954
Rathlev T & Pfau C J.: Purification of the pathogenic *Treponema pallidum* by density gradient centrifugation. Scand. J clin. Lab. Invest. *17* 130–134 1965
Reed W P & Albright E. L. Serum factors responsible for killing of shigella. Immunology *26* 205–215 1974
Schrohenloher R. E., Kunkel, H G & Tomasi T B. Activity of dissociated and reassociated 19S anti-γ-globulins. J exp. Med. *120* 1215–1229 1964
Wardlaw A. C., The complement-dependent bacteriolytic activity of normal human serum. I The effect of pH and ionic strength and the role of lysozyme. J exp. Med. *115* 1231–1249 1962.

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STUDIES ON THE *TREPONEMA PALLIDUM* IMMOBILIZING ACTIVITY IN NORMAL HUMAN SERUM

3 *The Kinetics of the Immobilization Reaction of Normal and Immune Sera*

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Hederstedt, B Studies on the *Treponema pallidum* immobilizing activity in normal human serum. 3 Kinetics of the immobilizing reaciton of normal and immune sera. Acta path. microbiol. scand. Sect C 84 142–147 1976

The influence of immobilizing antibody complement and lysozyme on the *T pallidum* immobilization reactions of normal and immune sera was studied. Lysozyme shortened the lag periods and increased the reaction rates of the reactions of normal and immune sera. At high concentrations of added lysozyme, variations in the concentrations of immobilizing antibody and complement, within a wide range, did not further influence the kinetics of the two reactions. Preincubation with lysozyme did not influence the treponemes in the following immune serum immobilization reaction provided the lysozyme was removed before the addition of antibody and complement. Normal serum was found to immobilize *T pallidum* more rapidly than immune serum. This was seen also if the reaction mixtures were almost identical, the only difference being the immobilizing IgM antibody involved in the normal and the IgG antibody involved in the immune serum reaction.

Key words *Treponema pallidum* immobilizing activity normal serum immune serum.

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The *T pallidum* immobilization reaction of human normal serum (NS) was found to be dependent on complement activated through the classical pathway and an immobilizing antibody of the IgM class (*Hederstedt* 1976) The immobilizing activity of immune serum (IS) is achieved by the joint action of an immobilizing antibody of the IgG class (*Laurell & Hederstedt* 1958 *Julian et al.* 1969) and complement.

The NS (*Hederstedt* 1976) as well as the IS reaction (*Metzger et al* 1961 *Müller et al* 1973) was enhanced by lysozyme

Human NS immobilized *T pallidum* more rapidly than syphilitic serum in the TPI reaction (*Hederstedt* 1974) suggesting that this difference in activity was due to different serum factors and/or a different mode of action of the serum factors involved in the reactions.

In the present study the kinetics of the NS and IS reactions were studied

MATERIALS AND METHODS

The normal sera (NS) were obtained from healthy persons while *immune sera (IS)* were obtained from patients treated for late manifestations of syphilis. The sera were stored unheated in 2 ml

aliquots in glass test tubes at −60° C until used. Unless otherwise stated, the immune serum was heated at 56 C for 30 minutes before testing.

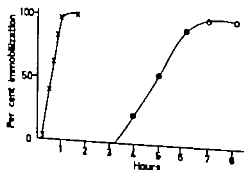
The normal ser (NS) wer freed of immobilizing antibody by absorption at 0° C with suspension of *T pallidum* purified by centrifugation, through density gradient (*Rathle & Pfau* 1965 *H derst dt* 1976) Following the centrifugation procedure, the treponemes were killed. The absorbed sera were used as sources of human complement. The haemolytic complement activity of an absorbed serum remained unaffected or was slightly reduced.

The lysozyme Hen egg-white lysozyme (Worthington Biochem. Corpor Freehold, N J) was used.

The T pallidum *immobilizing activity of normal ser* was determined as described by *Hederstedt* (1974)

The T pallidum *immobilizin g activity of immune ser (TPI test)* was determined as described by *Hederstedt & Skog* (1964) The reaction mixture was incubated under layer of paraffin oil containing 0 1 per cent of an oil soluble anti-oxidant, 2 6-ditert-butyl paracresol (*W ber* 1960)

The pretreatment of T pallidum *with lysozyme* To a suspension containing 4.5×10^7 treponemes per ml in Nelson medium (*Hederstedt* 1974) hen egg-white lysozyme was added to a final concentration of 200 µg per ml The mixture was incubated in N_2-CO atmosphere at 35 C for 18 hours. The incubated treponemes were washed twice with 100 parts PBS buffer pH 7 4 at +4 C and centrifugated in the cold at 20,000 *g* for 30 minutes. Before use in the immobilization reaction, the sedimented treponemes were resuspended in Nelson medium to concentration of 4.5×10^{6} treponemes per ml.



F g. 1 Immobilization at arious reaction times using normal human serum ×——× in dilution 1 5 6 and an immune human serum, ○——○, in dilution 1 5 0 The immobilizing (IT_{50}) and complement haemolytic (CH_{50}) titres of the normal serum were 15 6 and 163 units/ml, respectively The immobilizing titre of the syphilis serum was 320 and the haemolytic complement titre of the guinea pig serum used was 591 units/ml.

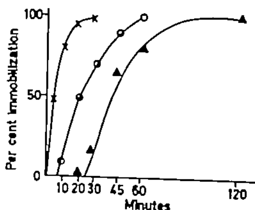
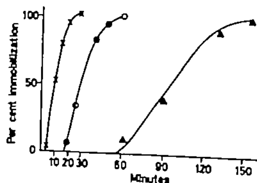


Fig 2 a.



F g 2 b

Figs. 2 and b Immobilization using normal (2 a) and an immune (2 b) human serum diluted 1:5.6 and 1:5.0 respectively at various reaction times and at arious concentrations of lysozyme. ×——× 40 µg of hen egg-white lysozyme added per ml of reaction mixture. ○——○ 4 µg of hen egg-white lysozyme added. ▲——▲ No hen egg white lysozyme added. The immobilizing titres of the sera were 12.9 and 460, respectively

RESULTS

The Kinetics of Normal and Immune Serum Immobilization Reactions

The rate of NS immobilization was greater and the lag period shorter than the rate and lag period of the IS reaction (Fig 1) The following factors were investigated to deter mine the mechanisms controlling these two reactions

(a) *Effect of lysozyme* The kinetics of the NS and IS immobilization reactions upon the addition of from 4 µg/ml to 200 µg/ml lyso-

zyme is shown in Figs 2 a and b The mobility of the treponemes was satisfactory even at the highest lysozyme concentration. The lag period of the NS as well as the IS reactions decreased and the reaction rate increased with increasing amounts of lysozyme up to around 20 μg The addition of >20 μg lysozyme did not bring about any further changes of the kinetics. The addition of 40 μg (to guarantee an excess of lysozyme) lysozyme per ml reaction mixture shortened the lag period of a NS reaction (the lag period was demonstrable at low serum concentrations only) from around 25 to less than five minutes and the lag period of an IS reaction from around 60–90 minutes to less than 10 minutes (Figs. 2 a and b) The acceleration of the immobilization of IS (as judged from the slopes of the curves in Figs. 2 a and b) appeared to be more pronounced than that of NS

(b) *Effect of immobilizing antibody* The NS and IS reactions were studied at various concentrations of immobilizing antibody and constant concentrations of haemolytic complement and lysozyme (40 μg lysozyme per ml reaction mixture) In the NS reaction the sera were diluted in their parent sera which were freed from immobilizing antibody thus keeping the concentration of complement almost constant. Within a wide range of NS and IS dilutions, 1 2 0 to 1 11 2 and 1 5 to 1 640 respectively the length of the lag period varied very little (Figs. 3 a and b) After the lag period, the rate of reaction appeared to be slower at the highest dilution of IS At this dilution however 100 per cent immobilization was not obtained within the time period studied

(c) *Effect of complement* Above a complement concentration range of 6 6 to 40 3 CH_{50} units per ml reaction mixture, the length of the lag period and the rate of the NS immobilization reaction did not vary (Fig 4 a) Using 6 6 units, however the immobilization reaction did not reach 100 per cent. Any difference in the IS kinetics of the immobilization reaction above a complement concentration range of 11.5 to 182 CH_{50} units was not observed (Fig 4 b) Using a complement concentration of six units no more than 40 per cent of the treponemes would be immobilized

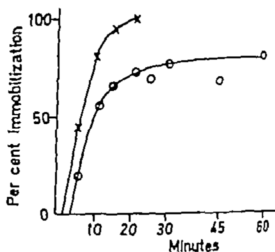


Fig 3 a

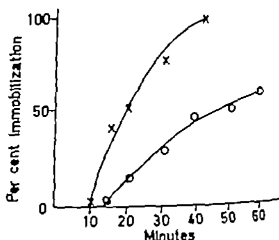


Fig 3 b

Figs 3 a and b Immobilization using a normal (3 a) and an immune (3 b) human serum at various reaction times and at various concentrations of immobilizing antibody 40 μg of hen egg-white lysozyme per ml of reaction mixture was added. ×——× normal and immune serum dilutions 1 2 0 and 1 5 0 respectively ○——○ normal and immune serum dilutions 1 11 2 and 1 640, respectively The immobilizing titres of the sera 14.2 and 1080 respectively

The Kinetics of the IS Immobilization Reaction with T pallidum *Pretreated with Lysozyme*

Treponemal suspensions were incubated with or without lysozyme for 18 hours. The

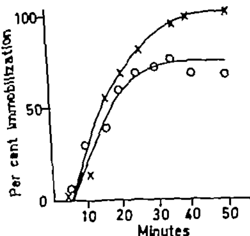


Fig 4 a.

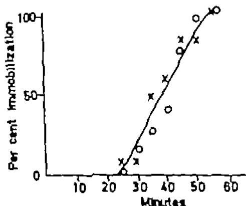


Fig 4 b

Figs. 4a and b. Immobilization using a normal (4a) and an immune (4 b) human serum, diluted 1:11.2 and 1:5.0, respectively at various reaction times and at various concentrations of complement. 40 μg of hen egg-white lysozyme per ml of reaction mixture was added. ×——× 40.3 and 182 CH_{50} units, respectively per ml of reaction mixture. ○——○ 6.6 and 11.5 CH_{50} units, respectively per ml of reaction mixture. The immobilizing titres of the sera were 20.2 and 900, respectively

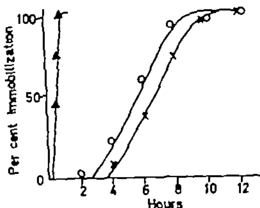


Fig. 5 The effect on the kinetics of the IS immobilization reaction if guinea pig serum was replaced by normal human serum as a source of complement. ×——× guinea pig serum (diluted 1:3) titre 189 CH_{50} units/ml. ○——○ normal human serum (freed from immobilizing antibody titre 210 CH_{50} units/ml. ▲——▲ normal human serum containing immobilizing antibody (titre 14.9) titre 221 CH_{50} units/ml. The immobilizing titre of the immune serum was 470

treponemes were washed, resuspended in Nelson's medium, and mixed with IS and guinea pig serum. The lag periods of the IS reactions containing lysozyme-treated or -untreated treponemes were similar. If the lysozyme was not washed off or if lysozyme was added after the 18-hour incubation period, the lag period was shortened from around 150 to <30 minutes.

The Kinetics of the Immune Serum Immobilization Reaction in the Presence of Human Complement

The kinetics of the immobilization reaction of IS were studied using three different kinds of sera as source of complement: guinea pig serum diluted 1:3 (189 CH_{50} units/ml), NS undiluted (221 CH_{50} units/ml) or NS freed from immobilizing antibody (210 CH_{50} units/ml). If NS freed of immobilizing antibody were used as complement, the kinetics of the IS reaction would be almost the same as those observed if guinea pig serum had been used (Fig. 5). If the NS containing immobilizing antibody were employed, the lag period would be reduced from about three hours to a few minutes and the reaction rate would increase.

The results illustrated in Figs. 1–5 were based on at least three different experiments. NS or IS from different human individuals were used in each experiment.

DISCUSSION

The lag period of IS was much longer, usually two to four hours longer, than that of NS, the latter being too short to be measurable at a high serum concentration (*Hederstedt 1974*) Moreover the reaction rate of NS was faster than that of the IS (Fig 1) These findings are in contrast to the statement in a paper by *Fribourg-Blanc* (1956) that the kinetics of the NS and IS immobilization reactions are identical

It has been suggested that the long lag period of the IS reaction is due to the time necessary for lysozyme (derived from the human serum guinea pig serum and rabbit tissue in the treponemal suspension) to remove a hypothetical protective" non-antigenic slime layer of capsule on the surface of *T pallidum* (*Christiansen* 1963, *Jones et al* 1968)

In fact, addition of extra lysozyme to the reaction mixture of the IS reaction shortened its lag period of from two–four hours to a few minutes and increased the reaction rate after the lag period.

At high concentrations of added lysozyme, variations in the concentrations of antibody and complement within a wide range did not further influence the kinetics of the IS reaction (Fig 2 b) This finding suggests that the different shapes of the curves of the NS immobilization reaction previously demonstrated (*Hederstedt* 1974) might be due rather to different amounts of lysozyme than to different amounts of complement or antibody in the NS of the different human individuals tested.

On the other hand the lag period of the NS reaction was very short even though the lysozyme content of the NS and IS reaction mixtures (no extra lysozyme being added) was approximately of the same magnitude, e.g the lysozyme *concentration in sera from* guinea pig and man was found to be 2.8–5 9 and 2.0–7 0 µg/ml, respectively (*Lundblad et al.* 1974)

Treatment of the treponemes with lysozyme prior to the addition of antibody and complement did not shorten the lag period of the IS reaction. The result is not in support of the postulation that the occurrence of a lysozyme-sensitive material covers the antigenic determinants of the treponemes. The latter is in agreement with the results obtained in similar studies presented by *Muller et al.* (1973)

The different kinetics of the two reactions may suggest that the NS and IS reaction mixtures constitute a homologous and heterologous system, respectively in other words, the complement and/or lysozyme from different species may be responsible for the different kinetics of the two reactions. If however the source of complement in the IS reaction were changed the compositions of the two reaction mixtures would be almost identical. This change did not give rise to any significant modification of the IS reaction rate (Fig 5) suggesting that the different kinetic behaviour of the two reactions was not due to species differences of the complement or lysozyme.

The main difference between the two reaction systems appeared to be the participation of the immobilizing IgM antibody in the NS reaction and the highly specific IgG antibody in the IS reaction. The activation of the amount of complement needed to immobilize the treponemes might be a process that is faster in the NS than in the IS system.

Since extra lysozyme added to the IS reaction mixture resulted in an immobilization reaction almost as fast as that of NS, it is possible that this enzyme could potentiate complement in the immobilization procedure In the present work this possibility was suggested by the finding that less complement, 6 6 to 11 5 CH_{50} units (Figs. 4 a and b) was needed to immobilize the treponemes at high concentrations of lysozyme

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REFFRENCES

Christiansen S Protective layer covering pathogenic treponemata. Lancet *i* 423–425, 1963.
Fribourg-Blanc A Le pouvoir tréponémicide naturel du serum humain Presse méd. *64* 1396–1397 1956

Hederstedt B., Studies on the *Treponema pallidum* immobilizing activity in normal human serum. I. A method. Acta path. microbiol. scand. Sect. B, *82* 185–200, 1974

Hederstedt B. Studies on the *T ponema pallidum* immobilizing activity in normal human serum. 2. Serum factors participating in the normal serum immobilization reaction. Acta path. microbiol. scand. Sect. C, *84* 135–141 1976.

Hederstedt B & Skog, E., Quantitativ *T ponema pallidum* immobilization (TPI) test in early syphilis. Acta derm. enereol. (Stockh.) *44* 82 90, 1964

Jones R. H Nevin T A., Guest W J & Logan, L. C Lytic effect of trypsin, lysozyme and complement on *Treponema pallidum* Brit. J ener Dis *44* 193–200 1968

Julian A. J Logan L. C & Norins, L. C Early syphilis immunoglobulins reactive in immunofluorescence and other serologic tests. Brit. J ener. Dis. *39* 30–32, 1969

Laurell, A. B & Hederstedt B., Fractionation of TPI antibodies and Wassermann reagins. Acta path. microbiol. scand. *44* 88–91 1958.

Lundblad G Hederstedt B., Lind J & Steby M.: Chitinase in Goat Serum. Preliminary purification and characterization. Eur J Biochem. *46* 367 376, 1974

Metzger M., Hardy P H Jr & Nell, E. E. Influence of lysozyme upon the treponeme immobilization reaction. Amer J Hyg. *73* 236–244 1961

Miller F., Feddersen H & S gerling, M., Studies on the action of lysozyme in immune immobilization of *T ponema pallid m* (Nichols strain) Immunology *24* 711–719 1973

Rathlev T & Pfau C. J., Purification of the pathogenic *T eponema pallid m* by density gradient centrifugation. Scand. J clin. Lab. Invest. *17*: 130–134 1965.

Weber M M., Factors influencing the *in vitro* survival of *Treponema pallidum*. Amer J Hyg. *71* 401–417 1960.

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STUDIES ON THE *TREPONEMA PALLIDUM* IMMOBILIZING ACTIVITY IN NORMAL HUMAN SERUM

4 The Importance for the Outcome of the Conventional TPI Test

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Hederstedt, B Studies on the *Treponema pallidum* immobilizing activity in normal human serum. 4 The importance for the outcome of the conventional TPI test. Acta path. microbiol. scand. Sect. C, 84 148–152 1976

T pallidum immobilizing activity was demonstrated in the majority (84 per cent) of samples of unheated sera obtained from healthy adult subjects. A similar activity was found in unheated sera obtained from some animal species such as monkey and white rat but not in sera from rabbit or guinea pig The results of serological studies of acquired, early syphilis in man suggested that the determination of the activity of the naturally occurring IgM immobilisins was not influenced by coexisting TPI antibodies of the IgG class. The immobilizing activity of human normal sera occurred in low titres only rarely in dilutions of >1 32 Moreover sera from guinea pig and rabbit were found to inhibit this activity in normal sera from man. These findings were supposed to explain why the immobilizing activity of the naturally occurring IgM antibodies did not interfere with the outcome of the conventional TPI test.

Key words *Treponema pallidum* immobilizing activity humanserum TPI test.

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The TPI test is the only bactericidal test which has found wide application in diagnostic immunology apparently because the occurrence in normal serum (NS) of natural antibodies will in most bactericidal reactions interfere with the specificity

The antibodies in immune serum (IS) reacting in the TPI test belong to the IgG class (*Laurell & Hederstedt* 1958 *Julian et al* 1969)

In a TPI test using a modified method (*Hederstedt* 1974) unheated human NS were found to possess *T pallidum* immobilizing activity The antibodies involved in the NS reaction were found to be of the IgM variety (*Hederstedt* 1976 a)

The purpose of the present study was to find out whether or not the NS immobilizing activity might influence the outcome of the conventional TPI test.

MATERIALS AND METHODS

The human normal sera (NS) were obtained and stored as previously described (*Hederstedt* 1974)

The human syphilitic sera (IS) were obtained from patients in venereal departments of hospitals in Stockholm. The sera were separated from the blood within ? hours after sampling and were stored in the same way as the normal sera.

The sera of various animal species were stored in the same way as the normal human sera.

The syphilitic patients (in hospital) were treated with 600,000 IE of procain penicillin daily for 7–21 days.

The T pallidum immobilizing activities of the normal sera and of the immune sera were determined as previously described (*Hederstedt* 1974, *Hederstedt & Skog* 1964). The serum titres were expressed as the reciprocal of the final serum dilutions to give 50 per cent immobilization and were referred to as the NS and the IS immobilizing titres, respectively. All the various samples from one and the same person or animal were titrated at the same time.

The inhibition of the T pallidum immobilizing activity of human NS by sera from guinea pig and rabbit. Equal amounts (0.125 ml) of twofold dilutions of normal unheated sera from guinea pig or rabbit (in the absence of immobilizing activity) and an undiluted normal human serum (with known immobilizing titre) were mixed and tested for immobilizing activity. The highest dilution of sera from guinea pig or rabbit in which sera < 20 per cent of the treponemes were immobilized was recorded.

Gel filtration of pools of sera from guinea pig or rabbit was carried out on Sephadex G-200 columns.

RESULTS

A Comparison of the Recorded NS Immobilizing Titres in Unheated Human Normal Sera (NS) and Early Syphilis Sera (IS)

Unheated sera from healthy female and male adults and from infants less than 12 months old were tested quantitatively for immobilizing activity. Immobilizing activity was demonstrated in 84 per cent of the sera from adults—82 per cent in female sera and 86 per cent in male sera—and in 91 per cent of the infant sera (Table 1). The highest titre recorded was 16.5. There was no significant difference in mean titres in the three different groups of sera.

All the NS were found to be non-reactive if tested by the conventional TPI test.

The mean NS immobilizing titres of sera taken prior to the treatment of patients with early syphilis were higher than those of sera from healthy persons while the titres of sera taken six months after treatment were at the same level as those of the normal sera (Table 2).

The Influence of the Immobilizing IgM Antibodies on the Development of Immobilizing IgG Antibodies in Sera from Patients with Syphilis at Early Stages

In cases of early syphilis, the IS titres in sera presenting high NS immobilizing titres were not found to be higher than those in sera presenting low NS titres (Table 3).

T pallidum *Immobilizing Activity in Normal Sera from Various Animal Species*

Sera from monkey, white rat, sheep and dog, but not sera from rabbit, guinea pig, hamster, ox, white mouse and goat possessed immobilizing activity (Table 4). In the cynomolgus monkey, the individual frequency of occurrence (74 per cent) and titre range of the serum activity were almost the same as that in man.

TABLE 1. *The T pallidum Immobilizing Activity in Unheated Sera from Healthy Individuals without History of Syphilis*

Sera	Number of sera	NS immobilizing titre		Frequency (%) of the NS immobilizing activity
		range	mean*	
Males	58	<2.0–13.2	3.9	86
Females	50	<2.0–16.5	4.2	82
Infants	34	<2.0–12.7	4.4	91

* In calculations of the mean titres, titre values of <2.0 were counted as 2.0

TABLE 2 *The NS Immobilizing Activity of Sera from Patients with Primary and Secondary Syphilis, prior to and after Treatment*

	Prior to treatment		2 months after treatment		6 months after treatment	
	Number of sera	NS immobilizing mean titre	Number of sera	NS immobilizing mean titre	Number of sera	NS immobilizing mean titre
Primary syphilis	119	6.2	51	4.6	43	4.0
Secondary syphilis	57	7.9	28	7.6	23	4.3

TABLE 3 *The Influence of the Naturally Occurring Immobilizins on the Development of TPI Antibodies in Early Syphilis*

Treatment started	NS immobilizing titre	Number of sera	IS immobilizing titre	
			range	mean
4–8 weeks after exposure	<7.4	21	<5–98	6.2
	>7.4	20	<5–160	8.0
9–12 weeks after exposure	<5.3	18	<5–180	12
	>5.3	17	<5–200	16
>12 weeks after exposure	<7.7	12	53–690	280
	>7.7	12	76–2590	190

TABLE 4 *The Occurrence of NS Immobilizing Activity in Various Animal Species*

Species	Number of sera	NS immobilizing titre	
		range	mean
Monkey (cynomolgus)	138	<2.0–16.3	7.1
Monkey (certipitecus)	28	5.4–18.6	10.2
White rat	35	7.1–15.4	11.9
Sheep	6	2.4– 3.9	2.8
Dog	3	5.9– 7.2	6.4
Rabbit	25	<2.0	<2.0
Guinea pig	25	<2.0	<2.0
Hamster	25	<2.0	<2.0
Ox	15	<2.0	<2.0
White mouse	25	<2.0	<2.0
Goat	3	<2.0	<2.0

TABLE 5 *The Inhibiting Capacity of Ten Sera from Guinea Pig and Ten Sera from Rabbit on the* T pallidum *Immobilizing Activity (Titre 1:14) of an Undiluted Human NS*

Source of sera	Number of animal sera, the highest dilution of which (mixed with an undiluted human NS) immobilized <20 per cent of the treponemes. Dilutions of the animal sera				
	Undiluted	1:2	1:4	1:8	1:16
Guinea pig	6	4	–	–	–
Rabbit	–	1	2	3	4

The Effect of Sera from Guinea Pig and Rabbit on the Immobilizing Activity of Unheated Normal Human Serum

The guinea pig and the rabbit sera exerted an inhibitory effect on the immobilizing capacity of the undiluted human NS (Table 5).

Following separation on Sephadex G-200, the inhibiting capacity of sera from guinea pig and rabbit was demonstrated exclusively in fractions from the 19 S peak.

DISCUSSION

In the reaction mixture used in the conventional TPI test, heated human serum is usually used in final dilutions of 1:5 or 1:10, guinea pig serum (as source of complement) being used in dilutions of 2:5 or 1:5. In the treponemal suspension, minute amounts of rabbit serum might occur.

In the present study where a modified TPI test was used, *T. pallidum* immobilizing activity was demonstrated in more than 80 per cent of unheated human NS. Sera from some animal species such as monkey and white rat, but not sera from guinea pig or rabbit, were also found to possess immobilizing activity.

In the human NS, an immobilizing activity was demonstrated in dilutions up to 1:16, suggesting that this naturally occurring activity might give rise to false positive reactions in the conventional TPI test. All the human NS thus tested, however, turned out to be negative. This negative outcome might be explained by the finding that unheated sera from guinea pig and rabbit exerted an inhibiting effect on the immobilizing activity of human NS.

A contributory cause of the negative outcome would be the presence of SH-compounds in the treponemal suspension used in the conventional TPI test (*Hederstedt* 1974). These compounds might inactivate the naturally occurring IgM immobilisins (*Deutsch & Morton* 1957).

T. pallidum immobilizing activity in NS from man and from various animal species was also observed by *Fribourg-Blanc* (1956) and *Müller & Segerling* (1969). Their observations, however, were not in full agreement with the results obtained in the present study. Thus, *Fribourg-Blanc* demonstrated weak reactivity in sera from rabbits, while *Müller & Segerling* did not find any immobilizing activity in sera from sheep and rat. This disagreement may be due to the different methods used.

The mechanism of the inhibition of the NS immobilizing activity caused by macroglobulin fractions of normal sera from guinea pig and rabbit is not clearly understood. The phenomenon, however, is probably not unique and may be analogous to e.g. the finding by *Herberman* (1970) that human IgM inhibited the natural cytotoxic rabbit antibody.

Results of serological studies of aquired early syphilis in man revealed that the titre of the immobilizing activity of the NS type increased after the syphilis infection had set in. Upon treatment, the NS titres appeared to decrease to their initial levels i.e. the mean titre in cases of treated, early syphilis was of the same magnitude as that in healthy persons.

In cases of early syphilis the development of TPI (IgG) antibodies did not appear to be influenced by the occurrence of NS immobilisins (Table 3). This may find its explanation in the fact that the immobilizing NS antibodies belong to the IgM class (*Hederstedt* 1976 a), the antibodies of which are known to give a poor anamnestic reaction. Moreover, the results of further studies have indicated that the specificity of the two types of antibodies apparently is different (*Hederstedt*, unpublished results).

It might be questioned whether the occurrence of IS antibodies would interfere with the determination of NS immobilizing activity in unheated syphilis sera. Since the NS and the IS antibodies apparently are directed towards different antigens (*Hederstedt*, unpublished results) and since the NS immobilizing titre is determined after incubation for two hours, while the lag phase of the IS immobilization reaction usually covers more than two hours (*Hederstedt* 1976 b), it appears to be possible to distinguish between the two.

This possibility is further supported by the finding that the NS immobilizing titre in sera from patients with syphilis had decreased six months after treatment to the same level as the mean titre of sera from healthy persons, irrespective of a presence or absence of IS immobilisins (Table 2)

The conclusion to be drawn is that the naturally occurring *T pallidum* immobilizing serum antibodies do not interfere with the outcome of the TPI test. The result of the present study does not change the common opinion that the TPI test is a most valid and specific test by which to establish the serological diagnosis of syphilis.

I wish to express my sincere gratitude to my colleagues at the venereal clinics of Karolinska Sjukhuset S t Görans Sjukhus and Södersjukhuset for their kind help and cooperation in the collection of the sera and the anamnestic data on the patients. This study was supported by grants from the World Health Organization

REFERENCES

Deutsch H F & Morton J I Dissociation of human serum macroglobulins. Science *125* 600 1957

Fribourg-Blanc A Le pouvoir tréponémicide naturel du serum humain. *Presse médicale 64* 1396–1397 1956

Hederstedt B. Studies on the *Treponema pallidum* immobilizing activity in normal human serum. 1 A method. Acta path. microbiol. scand. Sect. B *82* 185–200 1974

Hederstedt B Studies on the *Treponema pallidum immobilizing activity in normal human serum* 2 Serum factors participating in the normal serum immobilization reaction. Acta path. microbiol. scand. Sect. C, *84* 135–141, 1976 a.

Hederstedt B Studies on the *Treponema pallidum* immobilizing activity in normal human serum 3 A comparative study on the kinetics of the immobilization reaction by normal and immune sera. Acta path. microbiol. scand. Sect. C, 84 142–147 1976

Hederstedt B. Studies on the *Treponema pallidum* immobilizing activity in normal human serum 6. Antigenic relationship between *T pallidum* and various mammalian tissues. Unpublished results.

Hederstedt B & Skog E. Quantitative *Treponema pallidum* immobilization (TPI) test in early syphilis. *Acta derm. venerol. 44* 82 90, 1964

Herberman B Inhibition of natural cytotoxic rabbit antibody by human IgM Production of nontoxic rabbit serum for use as complement source J Immun. *104* 805–809 1970.

Julian A J., Logan L. C & Norins L. C. Early syphilis immuno globulins reactive in immunofluorescence and other serologic tests. Brit. J vener Dis. *39* 30–32, 1969

Laurell A B. & Hederstedt B. Fractionation of TPI antibodies and Wassermann reagin. Acta path. microbiol. scand. *44* 88–91 1958.

Müller F & Segerling M Comparative studies *on the immobilizing* and hemolytic complement activity in the sera of different species. WHO/VDT/RES 69 173 1969

EVALUATION OF AN IMMUNODIFFUSION TEST FOR SCREENING OF RUBELLA IMMUNITY

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Grandien, M., Espmark, A. & Norrby, E. E. Evaluation of an immunodiffusion test for screening of rubella immunity. Acta path. microbiol. scand. Sect. C, 84: 153–160, 1976.

Rubella antigen obtained by treatment with a mild neutral detergent (Brij-58) of rubella virus infected SIRC cells gave two lines of precipitate in a modified Ouchterlony test (ID) using sera collected during convalescence after rubella. One strong line was obtained with positive sera and sometimes one thin line as well. The presence of at least one line was taken as indicator of rubella immunity. The pattern in which reagents were arranged allowed all positive serum samples to give precipitates showing antibody identity with a rubella control precipitate. Among women in the fertile age screened for immunity 75 per cent gave precipitates by the ID test and thus were considered to be immune. This should be compared with the 83 per cent positivity found by means of the haemagglutination-inhibition (HI) test. As the HI test occasionally gives false results due to non-removable non-specific inhibitors, the ID test on basis of the specificity seems to be well suited to be used as a method for the selection of individuals to be vaccinated against rubella.

Key words: Rubella immunity; immunodiffusion.

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Immunodiffusion tests with rubella antigen has been described in several reports. In 1969 *LeBouvier* (5, 6, 7) reported how this technique might be used to determine antibody responses in acute and congenital rubella infections and response to vaccination. In preparations of rubella virus infected cells he found two main antigenic components, theta and iota. Neither the theta nor the iota antigen was identical with the haemagglutinin, but some findings suggested that the theta antigen could possibly be a subunit or a precursor of the haemagglutinin. A third antigen was later reported by *Le Bouvier* (6). The antibody against the theta component appeared soon after the emergence of haemagglutination-inhibition (HI) antibodies, i.e. around three days after onset of rubella. The anti-iota antibodies appeared later and titres continued to increase even some months after onset of disease. Five years after the rubella disease had terminated *Le Bouvier* could demonstrate anti-theta antibodies, but no anti-iota antibodies. The precipitins in serum were assumed to reflect the state of immunity

ty (6) and *Le Bouvier* found that the presence of anti theta antibodies in sera implied previous infection. Similar results were recently published by *Paul et al.* (11) and by *Cappel et al.* (2).

Schmidt & Styk (14) described the use of a more sensitive method, the micro-immunodiffusion variant elaborated in Ouchterlony's laboratory by *Wadsworth* (15, 16) in experiments with rubella antigen. They concluded that also whole virions participated in the precipitation reaction. *Salmi* (12) used a similar micro-modification of the ID test, but employed a rubella antigen produced by alkaline extraction of virus infected cells. Antibodies in rubella convalescent sera gave 3 lines of precipitation. Two of these were reproducibly identified, whereas the third line appeared only occasionally. In these tests the lines appeared relatively late, i.e. not earlier than two weeks after onset of disease. A fully developed precipitin pattern was not seen until about 4 months after the onset. Among *Salmi's* HI antibody positive sera obtained at random in a population without any recent history of rubella, the frequency of samples yielding positive rubella ID precipitates declined from 70 per cent in the age group 10–12 years to 29 per cent in the age group 17–22 years and to only 7 per cent in the age group 30–35 years. On the basis of these findings it was proposed that the immunodiffusion test did not involve any potential properties qualifying it to be included in the diagnostic routine.

In the search for a mild and efficient method by which to produce split viral antigens, *Booth & Norrby* (1) found that a neutral detergent, Brij 58* (Atlas Chemical Industries, USA), gave a high yield of readily diffusable rubella antigen. This finding prompted the present re-evaluation of the immunodiffusion test as a diagnostic tool.

The aim of this study was to investigate whether this new type of antigen preparation employed in a further modified immunodiffusion technique could be used in large scale screening tests for immunity against rubella. A micro-modification of the Ouchterlony double-diffusion test and the Booth-Norrby split rubella antigen were used throughout these antibody tests.

* Polyoxethylenacetyl ether

MATERIAL AND METHODS

Antigen. Antigen was prepared by extraction with Brij 58 of rubella infected SIRC (Serum Institute Rabbit Cornea) cells (9) as described by Booth-Norrby for rubella infected Vero cells (1).

SIRC cell cultures grown out as monolayers in Roux bottles with Eagle's minimum essential medium (MEM) + 10 per cent calf serum and antibiotics were inoculated with rubella virus (strain Judith) at a multiplicity of 1–3 $TCID_{50}$ per cell. Cultures were rocked for 1–2 hours at 37 C, upon which MEM with Earle's salts containing 1 per cent bovine serum was added. Cultures showed advanced cytopathic changes after incubation for 3 or 4 days at 37 C. At this stage cells were harvested by scraping off with rubber policeman into small volumes of phosphate buffered saline (PBS) pH 7.2–7.4, from which the cells were sedimented by low speed centrifugation. They were then treated with a 1 per cent Brij-58 solution in distilled water for 20 minutes at room temperature (0.5 ml Brij 58 solution to each Roux bottle of cells, i.e. about 20 million cells). Control antigen was prepared correspondingly from uninfected cell cultures. Each batch of antigen was controlled separately by ID tests against several sets of paired sera from patients in whom the rubella infection had been diagnosed by conventional methods; antigen preparations giving good precipitation reactions against rubella convalescent sera were pooled. Satisfactory antigen preparations were dispensed in vials and frozen at –20 C. They had a titre in complement fixation tests of 16 to 32.

Sera

1) *Single sera from 1000 women.* These sera were sampled consecutively in the diagnostic laboratory to which they had been sent for determination of immunity against rubella by the HI test.

2) Paired sera* from cases of clinical rubella, collected during the routine diagnostic activity. The diagnosis in the selected cases had been verified by HI as well as by CF tests.

3) Paired sera** from 26 women vaccinated against rubella showing sero-conversion by the HI test.

* Kindly provided by Dr. *Peet Tull*, Danderyd hospital, Stockholm.

** Eleven of these serum pairs were kindly provided by Dr. *Erik Lycke*, Department of Virology, Sahlgren's Hospital, Gothenburg.



Fig. 1. Plastic template with 9 seven-hole patterns resting on 0.2 mm agarose layer on 8 × 8 cm lantern slide. Note that pieces of nylon wire (0.2 mm in diameter) between the slide and the template are placed in each corner determining the height of the gel layer. Steel balls are placed in the wells of the template to prevent the gel from filling the wells.

Haemagglutination-inhibition (HI) and complement fixation (CF) tests. Microtechniques including the use of disposable microtitre plates were employed both in CF (13) and HI tests (3).

Immunodiffusion (ID) test. Before immunity testing by ID tests 2-fold concentration of sera was achieved by reducing the volume from 0.2 to 0.1 ml by absorption with one bead of Lyphogel per sample (Gelman Instrument Company). The ID test was carried out according to *Wadsworth* modification of the Ouchterlony test (14, 15). One part of 3 per cent agarose in distilled water was mixed with 2 parts of phosphate buffered saline, resulting in final ionic strength of 0.12 M. Melted agarose was poured in between a glass slide precoated with agarose in distilled water and a plastic template. The template rested on 4 pieces of a 0.2 mm thick nylon thread placed at its corners. As described by Wadsworth, the wells of the templates are cylindrical with a diameter of 3.5 mm whereas the bottom holes were only 2 mm in diameter. Steel balls were placed in the wells before the hot agarose was poured onto the slide to prevent the filling of wells with agarose from below (see Fig. 1). After the agarose had solidified, the steel balls were removed by the aid of magnet. The wells were inspected and any agarose which had entered was removed by suction.

A volume of 25 µl of reactant was filled into each well of the template. The tests were incubated for 72 hours in moist chambers at room temperature. After removal of templates, the plates were soaked in physiological saline and distilled water, dried with filter paper under pressure for 30 minutes and then stained with Coomassie brilliant blue.

The pattern of wells used and the application of reactants are illustrated in Fig. 2. Control serum was added to the central well. Virus antigen was added to wells in position 12 and 6 o'clock. The remaining 4 wells were used for serum specimens to be investigated.

For large-scale testing, 8 × 8 cm lantern slides allowing the simultaneous analysis of 36 serum specimens were used (Fig. 1).

RESULTS

Precipitating activity of antigen preparations. In order to control the properties of antigen preparations they were tested against paired sera from patients with verified rubella. Using convalescent sera, one or two precipitating lines were observed whereas early acute phase sera were negative. No reactions were obtained if antigen prepared from uninfected cells was used. The pattern of lines obtained with different convalescent sera was similar: a heavy and somewhat indistinct line and a thin and very distinct line which was located more closely to the antibody well. The former line was always present in tests of positive sera whereas the occurrence of the thin line varied depending

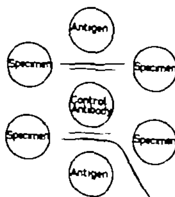


Fig. 2. Pattern used in immunodiffusion (ID) tests for rubella antibodies. In the centre well positive rubella antibody control. Above and below the centre well rubella antigen. Remaining wells are for serum specimens. A rubella antibody positive specimen is indicated in the well in position 4 o'clock.

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TABLE 1 *Appearance of Theta and Iota Lines Correlated to Day of Illness in Patients with Clinically and Serologically Verified Rubella*

Time of bleeding, days after onset of rash	Number of sera tested	Per cent of sera with theta line	Per cent of sera with iota line
1–3	6	0	0
4–5	16	62	0
6–8	10	100	0
15–18	14	100	43
19–22	20	100	85

TABLE 2 *Comparison of Results Obtained in Rubella Immunity Testings of 1000 Consecutively Collected Sera Using HI Tests and (after Two-fold Concentration) ID Tests*

HI titre	Total number of sera	Results of immunodiffusion tests		
		Positive	Negative	Identity uncertain
≥64	306	301	0	5
32	291	279	5	7
16	180	149	29	2
8	73	23	4[illegible]	3
<8	150	1§	141	8

* These reactions were in practice counted as negatives.

§ This serum had a titre of at least 5 in neutralization test against 200 $TCID_{50}$ of rubella virus.

upon the sera studied. There was a clear-cut distance between the two lines which allowed them to be readily distinguished by the naked eye in ordinary light after staining. These characteristics of the lines and their appearance in relation to time after infection (see below) made it clear that the heavy and thin lines corresponded to Le Bouvier's theta and iota lines, respectively.* The designations introduced by *Le Bouvier* will be used in the sequel.

When the paired sera from acute cases of clinically and serologically verified rubella (see Material and Methods) were tested it was found that the theta precipitate emerged 4–5 days after onset of symptoms and the iota precipitate about 2 weeks later (Table 1). All tests included a rubella convalescent control antiserum and antigen that gave the two-line pattern. The control serum was diluted so that the theta line of the control was located half way between the antigen and antibody wells, antigen preparations were generally used undiluted. Test sera were always placed adjacent to the serum control well in order that reaction of identity could be verified on positive sera. Occasionally precipitates were seen which did not show identity with the control lines (see Fig. 4 in the middle pattern to the right, position 8 o'clock). These sera were interpreted as negative for rubella antibody and further studies were not made to elucidate their significance. A typical pattern with positive antibody specimens is seen in Fig. 3. Fig. 4 shows the appearance of a slide from the routine test were the template contains 6 patterns of wells.

* As regards our standard reagents this was confirmed by Dr. *R. Cappel* (2), Institut Pasteur du Brabant, Bruxelles.

Immunity determinations by ID. One thousand consecutive sera from women (11–45 years of age) were analysed in HI anti-

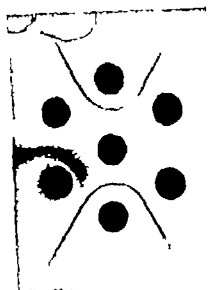


Fig 3 Example of positive rubella antibody reactions in immunodiffusion (ID) tests Arrangement of reactants as in Fig 2. All four sera have anti theta antibodies. In addition serum sample in position 10 o'clock contains anti iota antibodies.

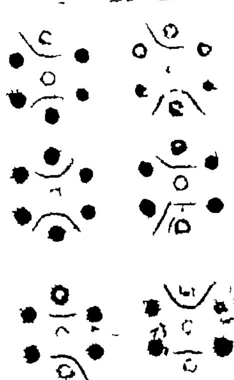


Fig 4 Results of ID tests for determination of rubella immunity Arrangement of reactants as in Fig 2. Six patterns of wells on the slide. Note the two non-specific precipitates of the rubella antibody negative serum in position 8 clock in the middle pattern to the right

body tests and, after two-fold concentration by Lyphogel absorption, in immunodiffusion antibody tests The results are presented in Table 2 There is a good correlation between presence of HI antibodies and positivity in the ID test. Among those with a HI-titre of <8, only one out of 150 was positive in ID This serum also contained neutralizing antibodies. In the groups with HI titres 8 or 16, 172 out of 253 were positive and finally among those with HI-titres in excess of 16, 580 out of 597 were positive (97 per cent) by the ID test.

Table 3 summarizes the frequencies of ID and HI positive sera from women in different age groups. In the whole material of 1000 sera, 75 per cent gave positive results by the ID test an incidence of 82 per cent positive ID test was found in the age group 31–35 years The corresponding percentage of sera judged positive by the HI test in the different age groups is given.

When the paired sera from rubella vaccinated women who showed seroconversion by the HI test were tested, 21 out of 25 also showed conversion by the ID test. Two women had precipitating antibodies before vaccination (HI titres 8) and two women were negative according to the ID test (HI titres 16) of the postvaccination serum.

DISCUSSION

In consideration of the prospective, steadily increasing use of rubella vaccination, the demands of high capacity methods for immunity testing are apparent. This does not apply only to the selection of candidate vaccinees, but later it may be found desirable to check the take-rate and perhaps the durability of immunity So far haemagglutina-

TABLE 3 *Frequencies of Sera Positive in ID and HI Tests among Samples from Women in Different Age Groups*

Age in years	Number of individuals tested	Per cent positive		
		ID test	HI test	
			Titre ≥ 8	Titre ≥ 16
11–15	11	(55)	(55)	(55)
16–20	167	72	80	75
21–25	340	79	87	80
26–30	298	75	84	76
31–35	111	82	90	82
36–40	40	75	90	82
41–45	33	61	85	63
	1000	75	85	77

tion inhibition tests (HI tests) have been used almost exclusively for such immunity testing

For determination of rubella immunity a situation in which the level of antibodies is of minor interest, the ID test offers several advantages over the conventional HI test. The most important of these is the readily visible identity of appearing precipitation lines. In fact, the double diffusion in gel is the only immunological test by which identity can be clearly observed with the naked eye To achieve a comparable accuracy in other tests the purity of the antigen must be beyond doubt, a requirement which only rarely can be fulfilled in practice False reactions due to non specific inhibitors represent a notorious problem in HI tests. Much attention has been paid to the development of absorption methods to be used in cases of these sera, but even the best of these do not guarantee the absence of non specific inhibitors in the HI test (4)

In recent years several efforts have been made to adapt the ID test to this purpose, but to our knowledge they have not in any case led to practical application for mass immunity screening

In the present study individual follow up of the ID results from serologically verified rubella cases was not done. Data on such cases are available only from 1968 and onwards, i e. a period of 7–8 years. This period of time is evidently too short for an evaluation of the longevity of ID lines. In years to come it is our plan to sample new sera from each year-group in order to study possible qualitative and quantitative changes of precipitation patterns in relation to time after primary infection. However rather firm indirect evidence indicates that precipitating rubella antibodies can be demonstrated for a considerable time. In fact, 82 per cent positive sera were found in the age group 31 35 years (cf Table 3) This suggests that rubella antibodies demonstrable by the ID test are sufficiently persistent to make the ID a useful practical test of immunity even among women of ages at the upper limit of childbearing This finding is in sharp contrast to the results obtained by *Salmi* (12) who found that the 70 per cent positivity in early convalescence after rubella very soon declined to low frequencies (7 per cent) thus invalidating the method as an immunity test. However *Salmi's* antigen was derived by alkaline extraction of cells (3) a procedure that might have extracted antigens to which only antibodies of short duration are formed.

The importance of including appropriate positive control reagents should be emphasized. For immunity testing only the theta line which is found to be more persistent than the iota line was considered of importance as an indicator of a past rubella infection. Great attention was paid to the position of this line in the control. When the control line was located mid way between the wells, it

was, as a rule, straight and without any deviations at the ends. Lines situated closer to one of the wells tend to deviate at the ends and thus may simulate a positive reaction. The function of the control line was primarily to establish the identity of precipitates caused by specimens. However it also served as an aid in the development of a possibly occurring weak precipitate—a well-known phenomenon.

Antibodies to rubella virus in different age groups in Sweden have earlier been determined by means of the neutralization test by *Landström et al* (10). In the age groups 15, 20, 25 and 30 years, 57, 74, 89 and 94 per cent, respectively of the individuals were found to have rubella antibodies. Altogether 83 per cent of women were found to transfer antibodies to their newborn babies. This tallies approximately with the frequencies of occurrence of antibodies demonstrable by the HI test. In our material of 1000 consecutive sera, 967 were derived from women in the age group of 11–40 years and 75 per cent of these were positive in the rubella ID test (Table 3).

If the ID test should be considered the sole test to be used for determination of immunity when individuals are to be selected for vaccination, it would bring about the inconvenience that 10 per cent of possibly seropositive women would be vaccinated; on the other hand this might eliminate the risk that women with false positive HI tests might be excluded from vaccination.

Le Bouvier & *Plotkin* (8) who studied a minor series of individuals who had been vaccinated with different rubella vaccines, claimed recently that the appearance of the iota line might indicate a relatively superior post-vaccination immunity. This suggestion evidently requires further confirmation through extended studies. In our studies, we neither divided the vaccinees into subgroups according to types of vaccine given, nor did we specifically enregister the appearance of iota lines. We read for theta lines just with a view to evaluating the test as a means by which the take-rate might be estimated, and in this respect the ID test seems to be useful, especially if its simplicity is taken into account. We believe that relative efficacy of different vaccines, including the duration of immunity has to be evaluated by other types of studies.

We are grateful to Dr *R. Cappel*, Department of Virology, Institut Pasteur du Brabant, for testing our reagents and confirming that our lines corresponded to the theta and iota lines described by *Le Bouvier*.

The excellent technical assistance of Mrs *Marianne Blomquist*, Mrs *Lena Sterne* and Miss *Anit Persson* is gratefully acknowledged.

REFERENCES

1. *Booth, J.* & *Norrby, E.* Preparation of rubella virus antigens for immunodiffusion by mild extraction of infected cells with a neutral detergent. Arch. Ges. Virusforsch. *41*: 132–134 1973.
2. *Cappel, R., Schluederberg, A.* & *Horstmann, D.* Large-scale production of rubella precipitinogens and their use in the diagnostic laboratory. J. Clin. Microbiol. *1*: 201–205 1975.
3. *Halonen, P. E., Ryan, J. M.* & *Stewart, J. A.* Rubella hemagglutinin prepared with alkaline extraction of virus grown in suspension culture of BHK-21 cells. Proc. Soc. Exp. Biol. Med. *125*: 162–167 1967.
4. *Haukenes, G.* A rubella haemagglutination inhibitor simulating antibody. Acta path. microbiol. scand. Sect. B, *81*: 719–723 1973.
5. *Le Bouvier, G. L.* Rubella precipitins. International symposium on rubella vaccines, London 1968; Symp. Series Immunobiol. Standard. *11*: 153–158 (Karger, Basel/New York) 1969.
6. *Le Bouvier, G. L.* Precipitinogens of rubella virus-infected cells. Proc. Soc. Exp. Biol. Med. *130*: 51–54 1969.
7. *Le Bouvier, G. L.* Physicochemical characteristics of rubella antigens *theta* and *iota*. Nature *221*: 78–79 1969.
8. *Le Bouvier, G. L.* & *Plotkin, S. A.* Precipitin responses to rubella vaccine RA 27/3. J. Inf. Dis. *123*: 220–223 1971.
9. *Leerhøy, J.* The influence of different media on cell morphology and rubella virus titer in rabbit cornea cell line (SIRC). Arch. Ges. Virusforsch. *19*: 210–220 1966.
10. *Landström, R., Sterner, A.*, *Höjer, L.* & *Keljner, K.*: Rubella immunity as related to age and history of overt disease. Acta Paed. Scand. *56*: 279–283 1967.
11. *Paul, V. R., Rhodes, A. J., Campbell, J. B.* &

TABLE 3 *Frequencies of Sera Positive in ID and HI Tests among Samples from Women in Different Age Groups*

Age in years	Number of individuals tested	Per cent positive		
		ID test	HI test Titre ≥ 8	HI test Titre ≥ 16
11–15	11	(55)	(55)	(55)
16–20	167	72	80	73
21–25	340	79	87	80
26–30	298	73	84	76
31–35	111	82	90	82
36–40	40	75	90	82
41–45	33	61	85	63
	1000	75	85	77

tion inhibition tests (HI tests) have been used almost exclusively for such immunity testing

For determination of rubella immunity a situation in which the level of antibodies is of minor interest the ID test offers several advantages over the conventional HI test. The most important of these is the readily visible identity of appearing precipitation lines. In fact, the double diffusion in gel is the only immunological test by which identity can be clearly observed with the naked eye. To achieve a comparable accuracy in other tests the purity of the antigen must be beyond doubt, a requirement which only rarely can be fulfilled in practice. False reactions due to non specific inhibitors represent a notorious problem in HI tests. Much attention has been paid to the development of absorption methods to be used in cases of these sera but even the best of these do not guarantee the absence of non specific inhibitors in the HI test (4)

In recent years, several efforts have been made to adapt the ID test to this purpose, but to our knowledge, they have not in any case led to practical application for mass immunity screening

In the present study individual follow-up of the ID results from serologically verified rubella cases was not done. Data on such cases are available only from 1968 and on wards i.e. a period of 7–8 years. This period of time is evidently too short for an evaluation of the longevity of ID lines. In years to come it is our plan to sample new sera from each year-group in order to study possible qualitative and quantitative changes of precipitation patterns in relation to time after primary infection. However rather firm indirect evidence indicates that precipitating rubella antibodies can be demonstrated for a considerable time. In fact 82 per cent positive sera were found in the age group 31–35 years (cf Table 3). This suggests that rubella antibodies demonstrable by the ID test are sufficiently persistent to make the ID a useful practical test of immunity even among women of ages at the upper limit of childbearing. This finding is in sharp contrast to the results obtained by *Salmi* (12) who found that the 70 per cent positivity in early convalescence after rubella very soon declined to low frequencies (7 per cent) thus invalidating the method as an immunity test. However *Salmi's* antigen was derived by alkaline extraction of cells (3) a procedure that might have extracted antigens to which only antibodies of short duration are formed.

The importance of including appropriate positive control reagents should be emphasized. For immunity testing only the theta line which is found to be more persistent than the iota line was considered of importance as an indicator of a past rubella infection. Great attention was paid to the position of this line in the control. When the control line was located mid way between the wells, it

THE MACROPHAGE RESPONSE IN MICE AFTER PRIMARY AND SECONDARY IMMUNIZATION WITH SHEEP RED BLOOD CELLS

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A primary and a secondary immune response to sheep red blood cells (SRBC) was evoked in mice by intraperitoneal injection of the antigen. Macrophage proliferation and migration as well as induction of the lysosomal enzyme acid phosphatase in macrophages were assessed along with lymphocyte proliferation. The proliferative responses were evaluated by ^{3}H-thymidine labelling (autoradiography). The secondary response elicited a faster and stronger lymphocyte proliferation than the primary response. This pattern was not seen to apply to the macrophages where the highest proliferative rate was found in connection with the primary response. The macrophages acquired an increased content of acid phosphatase during both types of responses, but the significantly highest level was reached in the secondary response. Large numbers of monocytes migrated into the peritoneal cavity during the first day after immunization. However, macrophage proliferation took place in resident as well as in immigrant cells.

Key words: Macrophage response; primary and secondary immunization; sheep red blood cells; mice.

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Macrophages play an important role at various stages of an immune response: in the induction phase where antigen is "presented" to the lymphocytes (25, 30) as well as in the effector phase of a cellular immune response (12, 13, 16, 20). A secondary immune response, as compared to the primary response, elicits typical alterations as regards the lymphocyte kinetics and the production of antibodies. However, whether a secondary reaction has corresponding consequences for various expressions of the macrophage response such as macrophage proliferation, induction of lysosomal enzymes, and macrophage migration, has not been thoroughly investigated. The present study was undertaken in order to elucidate this field. Furthermore, it has been proposed that macrophage activation (4, 9, 17, 28) and proliferation (18, 22, 26) are caused by products from stimulated lymphocytes. The in vivo relationship between different aspects of the macrophage response and the lymphocyte stimulation has therefore been investigated, mainly in an attempt to gain knowledge

Labzoffsky N A Rubella precipitin response in natural infection and in vaccination. Arch. Ges Virusforsch *45* 335–351 1974

12 *Salmi A A* Gel precipitation reactions between alkaline extracted rubella antigens and human sera. Acta path microbiol scand. *76* 271–278 1969

13 *Sever J L.* Application of a microtechnique to viral serological investigations. J Immunol *88* 320–329 1962.

14 *Schmidt N J & Styk B* Immunodiffusion reactions with rubella antigens. J Immunol *101* 210–216 1968.

15 *Wadsworth C.* A slide microtechnique for the analysis of immune precipitates in gel Int Arch Allergy *10* 355–360, 1957

16 *Wadsworth C* A microplate technique employing a gel chamber compared with other micro- and macroplate techniques for immune diffusion Int. Arch. Allergy *21* 131 151 1962

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about the mechanisms governing the changes in the macrophage population.

MATERIALS AND METHODS

Animals

Male NMRI/Bom mice aged 1–4 months, weighing 20–30 gram, were used in all experiments.

Immunization Procedure

A 50 per cent (v/v) suspension of sheep red blood cells (SRBC) in Baxter Alsever medium was adjusted to contain 10^9 SRBC/ml by dilution in 0.9 per cent NaCl. One ml of this suspension was injected intraperitoneally into each mouse to evoke a primary immune response. The same dose was used as challenge to provoke a secondary response in mice which had received 10^8 or 10^9 SRBC as a primary injection 4 weeks previously.

Harvesting of Peritoneal Cells

At different time points after elicitation of a primary or secondary immune response mice were killed and the peritoneal cells harvested. Cells destined for biochemical assays were harvested into medium 199 (Flow laboratories) containing 20 per cent foetal calf serum (Gibco), 50 I.U./ml of crystalline penicillin (Glaxo) and 50 µg/ml of streptomycin (Glaxo). Cells to be used for evaluation of DNA synthesis were harvested into the same type of medium containing in addition ^{3}H thymidine, 3 µCi/ml (specific activity 2 Ci/m-mole). Cells for biochemical assay were cultured on the bottom of Leighton tubes, while cells for autoradiography were cultured on 9 × 35 mm "flying cover slips" within the Leighton tubes. Further details have been given elsewhere (24, 25).

The cells were incubated at 37° C in an atmosphere of 5 per cent CO_2 in air. After 1½ hours, the cells for autoradiography were handled in the following manner. The Leighton tubes were agitated vigorously, the non adherent cells were harvested and the adherent cells were washed twice with 2 ml of medium 199. The non adherent cells from each Leighton tube were centrifuged, a drop of bovine albumin was added to the cell sediment, and smears were made. These smears as well as the cover slips containing the adherent cells were processed for autoradiography.

The cells for biochemical assay were cultured for one hour. The non-adherent cells were then discarded and the adherent ones were washed three times with 2 ml of 0.9 per cent NaCl. Thereafter the cells were wiped off the glass into 2 ml saline with a rubber policeman. These cell suspensions were stored at −20° C.

Autoradiography

Autoradiography was performed by way of the dipping technique using an Ilford L4 nuc. A film. After exposure for 7 days, the films were developed and stained with Giemsa. Cells with 5 or more grains overlaying the nucleus were scored as labelled. Three hundred cells were scored on each coded smear. The percentages of labelled cells of the respective cell populations are expressed as the labelling indices (L.I.).

Biochemical Assay

In order to destroy the cells and thereby activate the acid hydrolases, 10 repeated freezings and thawings were carried out.

Acid phosphatase (E.C.3.1.3.2.) was assayed with β-glycerophosphate as substrate (2) using an incubation period of 4 hours. The liberated phosphate was quantitated by the method of Fiske & Subbarow (6).

Protein was measured by the method of Lowry et al. using crystalline bovine serum albumin as a standard (15). More details have been given previously (25).

Colloidal Carbon Uptake

The resident phagocytic cells in the peritoneal cavity were labelled by injection of colloidal carbon (C 11/1432a, Gunther Wagner Pelikan, dilution 1:50). One week later SRBC were injected into the peritoneal cavities and on the subsequent first and second day the percentages of carbon labelled cells were estimated. The data obtained allowed an evaluation of the degree of macrophage influx to the peritoneal cavity. Autoradiography was also performed on such smears in order to ascertain whether DNA synthesis occurred in carbon-labelled, in non-labelled macrophages, or in both types.

Statistics

Examination of statistical differences was performed by the two-sided Wilcoxon test.

RESULTS

Cell Types

The free peritoneal cells of mice consist of lymphocytes and macrophages (8). After immunization with SRBC, granulocytes were found. Granulocytes adhered to the glass to a minor extent after culturing of the peritoneal cells and subsequent vigorous shaking of the Leighton tubes. Less than 5 per cent of the adherent cells were granulocytes. Most of the granulocytes were thus found among the non-

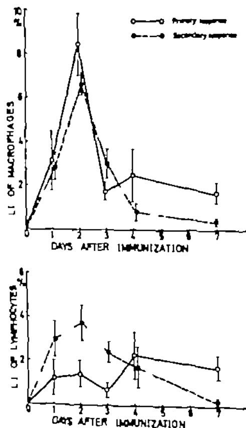


Fig 1 Labelling indices (L I) of peritoneal cells after primary and secondary immunization with SRBC. 1 A (upper fig) L I of macrophages. Each point represents the mean of values from 4-8 mice. Standard errors indicated by vertical bars. 1 B (lower fig) L I of lymphocytes from the same peritoneal cell populations as in Fig. 1 A

adherent cells. They constituted about 30 per cent of the non-adherent cells on day 1 and 2 after immunization and had decreased to about 10 per cent on day 7

Proliferation of Lymphocytes

The DNA-synthesis of peritoneal lymphocytes in a primary and a secondary immune response to SRBC was evaluated (Fig. 1B) In both situations the proliferative responses were rather low However the following conclusions can be drawn by comparing the two curves The DNA synthesis in the secondary response reached a maximum on day 2 (3.7 per cent) and was higher than that in the primary response during the first 3 days. The differences in DNA synthesis between the primary and the secondary response were statistically significant on each of these days ($p < 0.05$) In the primary response the highest value (2.3 per cent) was found on day 4 On day 7 there was still a low DNA synthesis rate present whereas that of the secondary response was negligible on that day

Proliferation of Macrophages

The labelling index applying to macrophages was found to be much higher than that applying to lymphocytes after immunization with SRBC (Fig. 1A and B) In the primary as well as in the secondary response the maximum L.I. was observed on day 2. A slightly higher L.I. was found in the secondary than in the primary response, 8.3 versus 6.6 per cent, but the difference was not statistically significant. In the secondary response the proliferation returned to near zero on day 7 and was thus less protracted than that in the primary response.

Acid Phosphatase of Macrophages

After immunization with SRBC, the acid phosphatase activity of macrophages, expressed as milliunits per mg cell protein, did not change in parallel with the changes in proli-

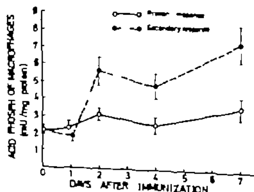


Fig 2 Acid phosphatase activity of peritoneal macrophages after primary and secondary immunization with SRBC Means ± SE are given. Values from 5–12 mice in each point.

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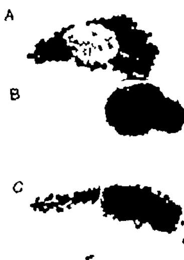


Fig 5 DNA synthesis in resident and immigrant macrophages. See text Figs. 3 and 4. *A and C* resident macrophages (containing carbon in their cytoplasm). C is DNA synthesizing (autoradiographic grains overlaying the nucleus) while A is not. *B* Immigrant macrophage (devoid of carbon) synthesizing DNA. Magn. × 1400.

first day No significant difference between the primary and the secondary immune response was found in this respect.

Proliferation of Resident or Immigrant Macrophages

In the experiments described above, autoradiography was also performed and the ^3H thymidine labelled macrophages were classified into two groups, those containing carbon and those without. Resident as well as immigrated macrophages were DNA-synthesizing (Fig 5) The carbon containing macrophages constituted 7 14 per cent of the DNA-synthesizing cells, while they constituted 13–21 per cent of the total macrophage population (Fig 4) This indicates that the proliferation rate of immigrated and resident cells is of the same magnitude. The slightly lower L I found in the latter group is not significantly different from the former

DISCUSSION

Opinions differ in the available reports on the manner in which the local tissue meets its requirement for macrophages, whether by local proliferation of resident macrophages (18, 21) or by influx of monocytes from the blood (14 29 31) In the present experiments it was clearly shown that large numbers of macrophages were supplied by influx of monocytes. However the data also showed that local proliferation took place, and that this proliferation was not confined to either resident or immigrant cells, but that both populations proliferated.

A number of changes can be induced in macrophages after exposure to lymphocyte mediators (lymphokines) Such mediators can inhibit macrophage migration, induce macrophage chemotaxis, increase the spreading of macrophages on glass, and cause aggregation as well as activation of macrophages (for review see 5) A lymphokine which is mitogenic for macrophages has been more difficult to demonstrate, probably because macrophages are reluctant to divide when adhered to glass or plastic surfaces. However *Godal et al.* (9) reported that the number of glass-adherent macrophages increased after addition of lymphocyte supernatants and, quite recently *Hadden et al.* (10) found DNA synthesis in adherent macrophages cultured in a medium containing such supernatants. In addition, two other groups (1 32) recently reported that they had induced macrophage proliferation *in vitro* by addition of inflammation exudate. A more exact nature of the mitogenic factor was not explored in those studies.

If macrophage proliferation *in vivo* were controlled by mediators from lymphocytes it would be reasonable to expect that the more marked lymphocyte stimulation in a secondary immune response, as compared with that of the primary response, would have consequences for the macrophage proliferation. The present experiments showed, however that a parallel to the more rapid and higher lymphocyte proliferation in the secondary

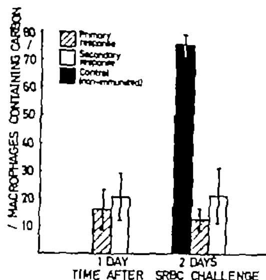


Fig 3 Influx of monocytes to the peritoneal cavities after SRBC immunization. Mice received colloidal carbon intraperitoneally. One week later the experimental mice were challenged with SRBC to give a primary or a secondary immune response, while control mice did not receive SRBC. On the *first and second day thereafter* the *proportions of* macrophages containing carbon were recorded

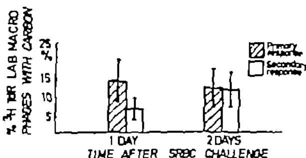


Fig 4 Proportions of ³H thymidine labelled macrophages containing carbon after SRBC challenge. See legend to Fig 3. The cover slips were submitted to autoradiography and ³H TdR labelled cells classified into two groups, those containing carbon and those without. The percentages of the former group are given. Vertical bars show ± 1 standard error

feration of lymphocytes and macrophages (Fig 2). Instead an increase was found throughout the 7-day observation period. This increase was only slight during the primary response, and much more pronounced during the secondary response.

The separated peritoneal macrophages had a significantly higher activity of acid phosphatase per mg protein than the total peritoneal cell population ($p<0.01$ Table 1). However the coefficient of variation for the data in the latter group was lower than that for the former 21 versus 37 per cent.

Influx of Macrophages to the Peritoneal Cavity

The macrophages of the peritoneal cavity were labelled by injection of colloidal carbon. One week later 76 per cent of the macrophages contained carbon particles in their cytoplasma (Fig 3). Macrophages with more than 4 particles were recorded as labelled. Usually it was not difficult to distinguish the labelled from the non labelled cells, since most of the labelled ones were heavily endowed with carbon. When animals which had been injected with carbon one week previously were challenged with SRBC to evoke a primary and a secondary immune response the percentages of carbon labelled macrophages dropped to about 15–20 per cent on the following day. The next day i.e. two days after challenge, the percentages were still of the same magnitude. The data show that there was a substantial influx of mononuclear phagocytes after SRBC injection and that the bulk of this influx occurred during the

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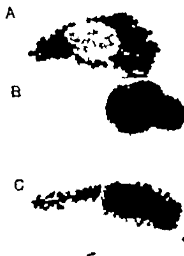


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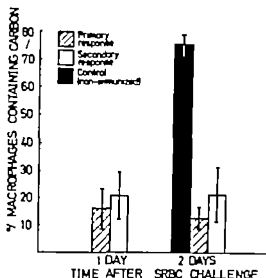


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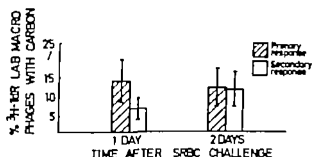


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5 *Bloom B R., Stoner G., Fischetti, V., Nowakowski, M., Muschel, R. & Rubinstein A.* Products of activated lymphocytes (PALs) and the virus plaque assay In Brent, L. and Holborow J (Eds) Progress in Immunology II Vol. 3 North-Holland, Amsterdam 1974 pp 133–144

6. *Fiske C H & S Subbarow Y* The colorimetric determination of phosphorus. J biol. Chem 66 375–400 1925

7 *Forbes I J* Mitosis in mouse peritoneal macrophages. J Immunol. 96 734–743 1966

8 *Furth, R. van & Cohn Z. A* The origin and kinetics of mononuclear phagocytes. J exp. Med. *128* 415–433, 1968.

9. *Godal, T Rees R. J W & Lamvik J O* Lymphocyte-mediated modification of blood-derived macrophage function in vitro inhibition of growth of intracellular mycobacteria with lymphokines Clin. exp. Immunol. *8* 625–637 1971

10. *Hadden J W Sadlik J R. & Hadden, E. M* Macrophage proliferation induced in vitro by lymphocyte factor Nature (Lond.) 257 483–485, 1975

11 *Hibbs J B jr* Heterocytolysis by macrophages activated by bacillus Calmette-Guérin Lysosome exocytosis into tumor cells. Science 184 468–471 1974

12. *Hibbs, J B. jr Lambert L. H & Remington J S* Possible role of macrophage mediated nonspecific cytotoxicity in tumour resistance. Nature (Lond) *235* 48–50 1972.

13 *Keller R & Jones V E.* Role of activated macrophages and antibody in inhibition and enhancement of tumour growth in rats. Lancet *ii* 847–849 1971

14 *Lala P K* Dynamics of leucocyte migration into the mouse ascites tumor Cell Tissue Kinet 7 293–304 1974

15 *Lowry O H Rosebrough, N J., Farr A. L. & Randall, R J* Protein measurement with the folin phenol reagent. J biol. Chem *193* 265–275 1951

16 *Mackaness G B* The immunological basis of acquired cellular resistance J exp. Med *120* 105–120 1964

17 *Mackaness G B* The influence of immunologically committed lymphoid cells on macrophage activity in vivo. J exp Med *129* 973–992, 1969

18 *Mier D G Penrose J M Kearney R & Nelson D S* Immunological induction of DNA synthesis in mouse peritoneal macrophages Int Arch Allergy *44* 611–630, 1973

19 *Neta J Poulter L W & Turk J L.* Effect of lymphocyte mediators on macrophages in vitro.—A correlation of morphological and cytochemical changes. Clin. exp. Immunol. *13* 453–466 1973

20. *Nelson D S.* Macrophages as effectors of cell-mediated immunity CRC Crit. Rev Microbiol. *1* 353–384 1972.

21 *North R. J.* Cellular kinetics associated with the development of acquired cellular resistance. J exp. Med. *130* 299–314 1969

22. *North R. J.* The mitotic potential of fixed phagocytes in the liver as revealed during the development of cellular immunity J exp. Med. *130* 315–326, 1969

23. *Pierce C. W Kapp J A., Wood D D & Benacerraf B.* Immune responses in vitro. X. Functions of macrophages. J Immunol. *112* 1181–1189 1974.

24 *Rutkiem A. & Hirby E. A.* Phagocytosis and microbicidal capacity of mouse macrophages non-specifically activated in vitro. Acta path. microbiol. scand. Sect. C, *83* 121–128, 1975.

25. *Rutkiem A., Grammeltvedt R. & Hirby E. A.* Activated mouse macrophages: morphology lysosomal biochemistry and microbicidal properties of in vivo and in vitro activated cells. Acta path. microbiol. scand. Sect. C, *83* 129–138, 1975.

26 *Rutkiem A* Macrophage proliferation during a lymphocyte transformation response A study of mouse peritoneal cells stimulated antigenically in diffusion chambers. J reticuloendoth. Soc. *18* 273–282 1975

27 *Rutkiem A* Macrophage proliferation and activation during *Toxoplasma gondii* infection in mice Relationship to lymphocyte stimulation. Acta path. microbiol. scand. Sect. C, *84* 124–130, 1976.

28. *Simon H B & Sheagren J N* Cellular immunity in vitro. I Immunologically mediated enhancement of macrophage bactericidal capacity J exp. Med. *133* 1377 1389 1971

29 *Spector W G & Ryan G B* The mononuclear phagocyte in inflammation In: van Furth, R. (Ed.) Mononuclear phagocytes. Blackwell Scientific Publications, Oxford 1970 pp. *219–232.*

30 *Unanue E. R.* The regulatory role of macrophages in antigenic stimulation. Adv Immunol. *15* 95–165, 1972.

31 *Volkman A* The origin and turnover of mononuclear cells in peritoneal exudates in rats. J exp. Med. *124* 241 253 1966.

32 *Wynne K. M Spector W G & Willoughby D A.* Macrophage proliferation in vitro induced by exudates. Nature (Lond.) *253* 636–637 1975

response could not be retraced in the corresponding macrophage proliferation It is to be noted, however that a rapidly starting intraperitoneal macrophage proliferation might be initiated merely by repeated puncturing of the peritoneal cavity (7) or by injection of saline (27) In the latter study (27) it was shown that this macrophage proliferation occurred in the absence of any demonstrable lymphocyte stimulation. This indicates that macrophage proliferation under certain circumstances may be initiated in a principally different way i.e by non-immunological factors. If such mechanisms were operating in the present experiments, as they might have been, it could provide an explanation as to why macrophage proliferation in the secondary response did not differ from that found in the primary response Another possibility is that the considerable numbers of granulocytes during the first days could somehow have interfered with the macrophage proliferation. However we have not yet any exact information about such interactions.

The separation of macrophages from the total peritoneal cell population was based on the adhesive properties of the cells The acid phosphatase values recorded in separated macrophages showed considerable variation the individual data having a significantly higher coefficient of variation than the corresponding values found to apply to the total non-separated cell population. The reason for this is not evident, but the possibility might be considered that a variable degree of exocytosis of lysosomal enzymes could have occurred during the process of separation Such exocytosis has, to my knowledge, not been reported in experiments similar to the present one, but has been found in other situations (11) However even if some variable degree of exocytosis could have interfered, one would not expect that this should systematically have influenced the differences between different groups of macrophages separated in the same way Furthermore, the mean acid phosphatase levels were considerably higher in the separated macrophages than in the total peritoneal cell population showing that the former cells had indeed a high content of the enzyme.

Bearing the above mentioned reservation in mind, the results can be further discussed. Firstly the data showed that the increase in the cell content of acid phosphatase did not take place in parallel with the changes in cell proliferation. The changes in the former parameter were considerably retarded as compared with those in the latter Secondly the enzyme level in the cells reached during the secondary immune response was significantly higher than the level reached during the primary response. This increased enzyme level could be due to either a stimulation by mediators released from lymphoblasts (19) or to a more effective phagocytosis, since phagocytosis of digestible particles per se can result in induction of lysosomal enzymes (3). The latter mechanism was in any case likely to be operative in the present situation because the secondary response probably provided more antibodies against SRBC, thereby causing a more effective opsonization and phagocytosis.

The skilful technical assistance of *Eric Teogood* is gratefully appreciated.

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REFERENCES

1 *Adolphe M Fontagne J Pelletier M & Giroud J P* Induction of DNA synthesis in rat macrophages in vitro by inflammatory exudate Nature (Lond.) *253* 637 1975.

2 *Appelmans F Wattiaux R & DeDuve C.* Tissue fractionating studies. 5 The association of acid phosphatase with a special class of cytoplasmic granules in rat liver Biochem J *59* 438–445 1955

3 *Axline S G & Cohn Z A* In vitro induction of lysosomal enzymes by phagocytosis J exp. Med *131* 1239–1260 1970

4 *Blanden R V* Modification of macrophage function. J reticuloendoth. Soc. *5* 179–202, 1968.

the clinical diagnosis of yersiniosis is often overlooked, and thirdly the heterologous titres observed in the cross-reaction are equal (or almost equal) to the homologous titres. This has resulted in an increased risk of overdiagnosis of brucellosis.

Recently an enzyme-linked immunosorbent assay (ELISA) (1–5) for quantitation of antibodies was described and found to be more sensitive than commonly used methods. The object of the present study was to investigate the usefulness of ELISA for detection and quantitation of antibodies against cell envelope antigens of species belonging to *Brucella* and *Yersinia*.

MATERIAL AND METHODS

Bacterial strains and preparation of lipopolysaccharides (LPS). *B. abortus* strain 544, *Y. enterocolitica* O-group V strain MY 79, *Y. enterocolitica* O-group I, subgroup A (previously designated serotype O-3) strain 482 and *Salmonella typhi* T2 came from the strain collection at the Department of Bacteriology, National Bacteriological Laboratory.

The *Brucella* and *Yersinia* strains were cultivated in Roux flasks with meat extract agar as described earlier (6). The bacteria were killed by adding formalin to a final concentration of 1 per cent; the whole cells were then extracted with hot phenol-water (7, 8). For *B. abortus* phenol phase LPS was used for coating of the tubes in ELISA, for *Y. enterocolitica* and *S. typhi* T2 aqueous phase LPS was used for coating (7, 8).

The *Salmonella* bacteria were grown in submerged culture (11) and harvested by centrifugation, killed by gamma irradiation and washed twice in phosphatebuffered saline (PBS) (pH 7.3). Bacterial cell walls were prepared as described earlier (11) and the LPS was extracted by the hot phenol-water method (12). The LPS preparations were purified by ultracentrifugation (105,000 × g) until no specific absorbance at 260 nm could be detected. If necessary, remaining ribonucleic acid was eliminated by treatment with ribonuclease (5 × Crystalline, Nutritional Biochemical Corp., Cleveland, Ohio, USA) as described before (8).

Antigens for Immunisation

One volume of washed and packed cells was suspended in four volumes of distilled water. Three volumes of chilled acetone were added to one volume of cell suspension. The mixture was stored overnight (18 h) at −20 C and the cells were then washed three times in cold acetone and dried in a desiccator over $CaCl_2$.

Antisera

Rabbits of both sexes (2.5–3 kg) were immunised with 10 mg acetone-killed bacteria (approximately 10^9 cells) diluted in 1 ml PBS and emulsified in one ml of Freunds incomplete adjuvant (Difco Laboratories, Detroit, Michigan, USA). Of the emulsion 1 ml was given subcutaneous twice weekly for seven weeks and the rabbits were bled one week after the last injection. Three to five rabbits were immunised with each *Brucella*, *Yersinia* and *Salmonella* strain. The sera were pooled and stored at −20 C until used.

Bacterial Whole Cell Agglutination

Serial two-fold dilutions of serum starting at 1:20 were prepared in PBS. Tubes containing 0.25 ml of serum and 0.25 ml of the antigen suspension were incubated overnight (18 h) at 52° C. The agglutination was read with the naked eye and the titre was determined as the highest serum dilution giving visible agglutination. All titrations were carried out twice in independent experiments.

Enzyme Conjugated Anti-immunoglobulin

Antibodies against rabbit immunoglobulin were obtained by immunospecific purification of hyperimmune sheep antiserum on insolubilized rabbit IgG. A 0.5 mg amount of anti-rabbit immunoglobulin was conjugated with 1.5 mg of alkaline phosphatase (calf intestinal mucosa, type VII, Sigma Chemical Co., St. Louis, Mo. USA) by the addition of glutardialdehyde (3). Conjugated material was separated from unconjugated enzyme and immunoglobulin by gel filtration on Sepharose 6B (Pharmacia AB, Uppsala, Sweden). The conjugate was stored at 4 C in 3 per cent human serum albumin (AB KABI, Stockholm, Sweden) in 0.05 M Tris HCl (pH 8.0) containing 0.02 per cent NaN_3.

Enzyme-linked Immunosorbent Assay

Titration of antibodies. ELISA was performed essentially as described by *Engvall & Perlmann* (4). Disposable polystyrene tubes (11 by 55 mm, Heger Plastics AB, Stallarholmen, Sweden) were incubated with 1 ml of LPS solution containing the appropriate amount of LPS from the bacterial strains under study in 0.05 M carbonate buffer (pH 9.6) containing 0.02 per cent NaN_3, for 3 h at 37° C. The tubes were washed three times with 0.9 per cent NaCl containing 0.05 per cent Tween 20 (Kemi Intressen AB, Sundbyberg, Sweden) before incubation with serum. In the washed LPS-coated tubes, 1 ml of serum diluted in PBS with 0.05 per cent Tween 20 and 0.02 per cent NaN_3 (PBST) was incubated for 3 h at room temper-

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ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR TITRATION OF ANTIBODIES AGAINST *BRUCELLA ABORTUS* AND *YERSINIA ENTEROCOLITICA*

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Carlsson, H. E. Hurvell, B & Lindberg, A. A Enzyme-linked immunosorbent assay (ELISA) for titration of antibodies against *Brucella abortus* and *Yersinia enterocolitica*. Acta path. microbiol scand Sect. C, *84* 168–176 1976

An enzyme linked immunosorbent assay (ELISA) using phenolwater extracted lipopolysaccharides as antigen was used for detection and quantitation of antibodies against *Brucella* and *Yersinia* bacteria in rabbit antisera. ELISA was found to be from ten to hundred fold more sensitive than the commonly used tube agglutination assay (Widal) In addition, both direct and inhibition assays using ELISA revealed antigenic differences between *Brucella abortus* and *Yersinia enterocolitica* O-group V previously undetected in tube agglutination and complement fixation studies. These data raise the possibility of a sensitive and specific assay for detection of anti *Brucella* antibodies in human sera.

Key words Enzyme-linked immunosorbent assay antibody titration *Brucella abortus* *Yersinia enterocolitica*.

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The clinical diagnosis of brucellosis is often difficult to establish because many infections are atypical, *i.e* the classical undulant fever is rarely seen. The incidence of positive blood cultures is low and the laboratory diagnosis is therefore mainly based on the detection and titration of *Brucella* antibodies in serum.

In Sweden, the incidence of human brucellosis is very low mainly because bovine brucellosis was eradicated in 1957 Sera from patients with an undiagnosed febrile illness are however still frequently submitted to the laboratory for investigation of anti-*Brucella* antibody titres. This is especially the case when the patient has a history including travelling to areas with brucellosis. The discovery of a serological cross-reactivity between *Brucella abortus* and *Yersinia enterocolitica* O-group V (previously designated serotype O-9) (10 13) complicated the serodiagnostic work (for references see 7 8, 9) First the incidence of yersiniosis is much higher than that of brucellosis (13) secondly

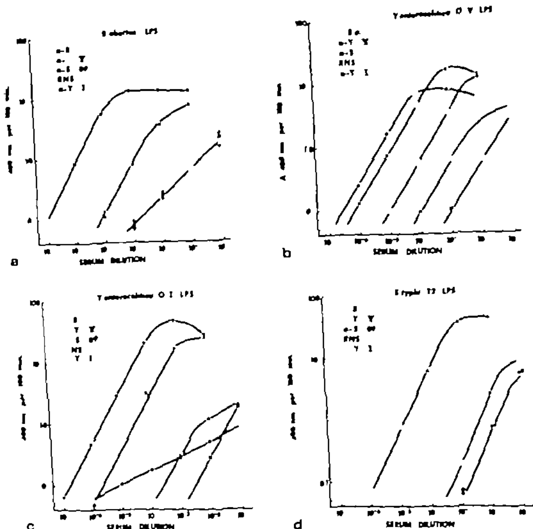


Fig 2 Titration of rabbit sera in ELISA against tubes coated with (a) 10 ng per ml of LPS from *B abort* (b) 5,000 ng per ml of LPS from *Y enterocolitica* O-V (c) 5,000 ng per ml of LPS from *Y enterocolitica* O-I and (d) 5,000 ng per ml of LPS from *S typhi* T2. Sera: anti-*B abortus* ●; anti-*Y enterocolitic* O-V × anti-*Y enterocolitica* O-I ▼ anti-Salmonella O-9 △ and RNS □.

higher titre (70,000) than the homologous *Y enterocolitica* O-V antiserum (50,000) again indicating a strong cross-reaction between the *B abortus* and *Y enterocolitica* O-V LPS (Fig 2b) Also the *Y enterocolitica* O-I antiserum showed a relatively high titre (2,000) indicating a cross-reaction between the two *Yersinia* species. However the *Salmonell* O-factor 9 antiserum and RNS gave low titres (100 and 20 respectively) against the LPS from *Y enterocolitica* O-V

When the pooled rabbit sera were tested against LPS from *Y enterocolitica* O-I as the antigen antisera against *Y enterocolitica* O-I and O-V gave high titres (150,000 and 10,000 respectively) in ELISA (Fig. 2c) This verifies the cross-reactivity observed in the reciprocal system. A low titre was seen with *B. abortus* antiserum (50) *Salmonella*

ature The tubes were washed as before, and 1 ml of sheep anti-rabbit Ig-alkaline phosphatase conjugate diluted 1 500 in PBST was added. After incubation overnight (18 h) at room temperature, the tubes were washed as above and 1 ml of a solution of enzyme substrate, p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo., USA) in 0 05 M carbonate buffer (pH 9 8) containing 10^{-3} M $MgCl_2$ was added. The enzyme reaction was performed at room temperature for 100 minutes or until the absorbance at 400 nm reached approximately 0 8 The reaction was stopped by the addition of 0 1 ml of 1 1 M NaOH

Inhibition studies A suboptimal dilution of antiserum in PBST (giving an absorbance of about 2 at 400 nm per 100 minutes in the final test) was added to antigen coated tubes together with an equal volume (0.5 ml) of different concentrations of LPS inhibitor The experiments were run in duplicate Maximum absorbance was determined in controls with PBST instead of the LPS inhibitor Tubes were incubated (5 h) washed and 1 ml of sheep anti-rabbit Ig-alkaline phosphatase diluted as above was added. The assay was then performed as described for titration of antibodies.

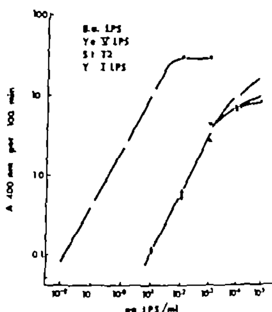


Fig 1 Titration of LPS concentration for optimal coating of plastic tubes in ELISA. The tubes were incubated for three hours with varying concentrations of LPS from *B abortus* ● *Y enterocolitica* O-V × *Y enterocolitica* O-I ▼ and *S typhi* T2, △ in 0 05 M carbonate buffer (pH 9.6) at 37° C. The coated tubes were then tested against the homologous hyperimmune antiserum diluted 1 10 000

RESULTS

Optimation of LPS concentration for coating of plastic tubes in ELISA

Plastic tubes were incubated with solutions of LPS from *B abortus* *Y enterocolitica* O V *Y enterocolitica* O I and *S typhi* T2 at different concentrations (Fig 1) There was a thousandfold difference in coating efficiency between LPS from *B abortus* (good sensitization with 1 to 100 ng LPS per ml) and LPS from the other three bacterial strains (100 to 10 000 ng) In the titration experiments 10 ng per ml of *B abortus* LPS and 5 000 ng per ml of LPS from *Y enterocolitica* O-V *Y enterocolitica* O-I and *S typhi* T2 were used for sensitization of the tubes.

Titration of Rabbit Sera in ELISA

Pooled rabbit antisera against *B abortus* *Y enterocolitica* O-V *Y enterocolitica* O I and as controls anti-*Salmonella* O factor 9 as well as pooled rabbit normal serum (RNS) were titrated against tubes coated with LPS. All samples were run in duplicates. The duplicates rarely deviated more than 5 per cent from the mean on the linear parts of the titration curves.

Figure 2a shows the titration of rabbit hyperimmune sera against LPS from *B. abortus* It can be seen that the pooled homologous antiserum gives by far the strongest reaction. Using the serum-dilution that gives an absorbance of 2.0 at 400 nm per 100 min as titre the *B abortus* antiserum shows a hundredfold higher titre (300 000) than the *Y enterocolitica* O-V antiserum which in turn gave a more than hundredfold higher titre (3 000 against *B abortus* LPS than the *Y enterocolitica* O-I antiserum (<10) the *Salmonella* O-factor 9 antiserum (10) and RNS (10) The cross-reaction between the *Y enterocolitica* O-V antiserum and *B. abortus* LPS was expected, but the homologous and heterologous titres differed significantly

When the five sera were tested against tubes coated with LPS from *Y enterocolitica* O V the *B. abortus* serum gave a slightly

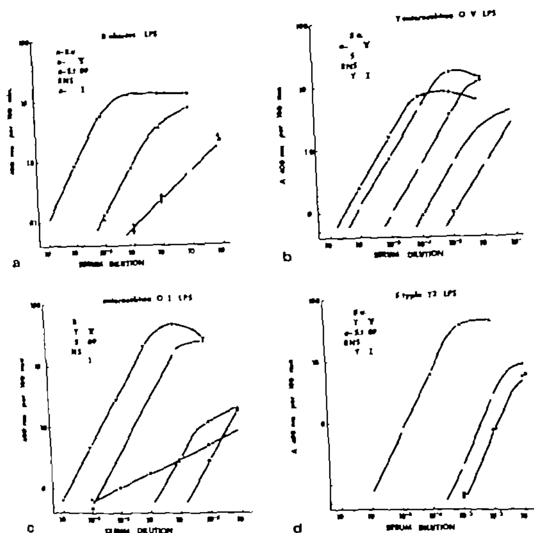


Fig 2 Titration of rabbit sera in ELISA against tubes coated with ( ) 10 ng per ml of LPS from *B abort* (b) 5,000 ng per ml of LPS from *Y enterocolitica* O-V ( ) 5,000 ng per ml of LPS from *Y enterocolitic* O-I and (d) 5,000 ng per ml of LPS from *S typhi* T2. Sera anti-*B abortus*, ●; anti-*Y enterocolitica* O-V × anti-*Y enterocolitica* O-I ▼ anti-Salmonella O-9 △ and RNS □.

higher titre (70,000) than the homologous *Y enterocolitica* O-V antiserum (50,000) again indicating a strong cross-reaction between the *B abortus* and *Y enterocolitica* O-V LPS (Fig. 2b) Also the *Y enterocolitica* O-I antiserum showed a relatively high titre (2,000) indicating a cross-reaction between the two *Yersinia* species. However the *Salmonella* O-factor 9 antiserum and RNS gave low titres (100 and 20 respectively) against the LPS from *Y enterocolitica* O-V

When the pooled rabbit sera were tested against LPS from *Y enterocolitica* O-I as the antigen antisera against *Y enterocolitica* O-I and O-V gave high titres (150,000 and 10,000 respectively) in ELISA (Fig. 2c) This verifies the cross-reactivity observed in the reciprocal system. A low titre was seen with *B. abortus* antiserum (30) *Salmonella*

TABLE 1 *Titres of the Different Rabbit Sera Tested in ELISA against LPS and in Agglutination against Whole Cells from the Four Different Bacterial Strains under Study*

Antigen	Serum					
	anti *B abortus*	anti *Y enterocolitica* O-V	anti *Y enterocolitica* O-I	anti *S typhi* O-9	RNS	Test system
Brucella abortus	2,560	640	20	20	20	Agglutination
	300 000*	5 000	10	10	10	ELISA
Yersinia enterocolitica O-V	2,560	2,560	20	20	20	Agglutination
	70 000	30 000	2,000	100	20	ELISA
Yersinia enterocolitica O-I	20	20	1,280	20	20	Agglutination
	30	10 000	150 000	10	10	ELISA
Salmonella typhi T2	20	20	20	640	20	Agglutination
	30	100	30	30 000	30	ELISA

* The titre in ELISA is defined as the serum dilution that gives an absorbance of 2.0 at 400 nm per 100 minutes.

O factor 9 antiserum (10) and RNS (10) against *Y enterocolitica* O-I LPS The slope of the titration curve of *B abortus* antiserum however differed significantly from the slopes of the other sera.

All sera were also titrated against tubes coated with LPS from *S typhi* T2 (*Salmonella O* factors 9 and 12) as a non related antigen control (Fig 2d) Only the homologous *Salmonella* O factor 9 antiserum gave a high titre (30 000) roughly thousand times higher than the other sera (≤100) Thus, as expected no cross-reactions between *S typhi* T2 LPS and antisera against *B abortus* *Y enterocolitica* O V or O I were detected

The titrations performed with ELISA and conventional whole cell agglutination are summarized and compared in Table 1 It was found that RNS gave a low titre (≤30) against all LPS antigens used. The *Salmonella* factor O 9 gave a high titre only against the homologous antigen The *Y enterocolitica* O I antiserum gave against LPS from *Y enterocolitica* O V an elevated titre in ELISA but not in tube agglutination The reaction against the homologous antigen was strong in both tests 150 000 in ELISA and 1,280 in tube agglutination. The *Y enterocolitica* O V antiserum gave high titres in both systems against *B abortus* LPS and homologous antigen, but only in ELISA against the LPS of *Y enterocolitica O I* The known cross-reaction between *Y enterocolitica* O V and *B abortus* was also seen with antiserum to *B abortus* in both ELISA and tube agglutination.

Inhibition of ELISA with LPS

The capacity of the four LPS species to inhibit the reaction between anti *B abortus* and homologous or *Y enterocolitica* O-V LPS, anti *Y enterocolitica* O-V and homologous or *B abortus* LPS as well as anti-*Y enterocolitica O-I* and homologous LPS was tested using ELISA (Fig 3 Table 2)

B abortus LPS was a thousand times more efficient than *Y enterocolitica* O-V LPS to inhibit the binding of anti *B abortus* antibodies to tubes coated with homologous or *Y enterocolitica* O-V LPS (Fig 3) The inhibiting power of LPS from *Y enterocolitica* O I was from six to ten times less efficient than that of LPS from *Y enterocolitica O-V* When anti *Y enterocolitica* O V serum was used in tubes coated with *B abortus* LPS there was an even greater difference in inhibition between the LPS preparations from *B abortus* and *Y enterocolitica O-V* *B abortus* was about 17 000 times more powerful as inhibitor LPS from *Y enterocolitica* O-I but was about four times less efficient compared to *Y enterocolitica* O-V LPS.

In the homologous system anti *Y entero-*

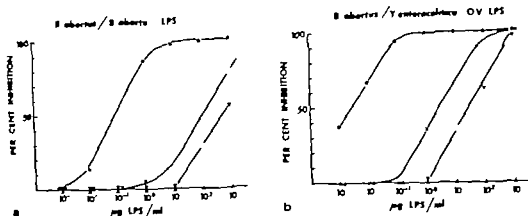


Fig 3 Inhibition by lipopolysaccharides of the binding of anti-*B. abortus* antibodies to tubes coated with, ( ) *B abortus* LPS, and (b) anti-*B. abortus* antibodies to tubes coated with *Y enterocolitica* O-V LPS In the anti-B a./B LPS system ( ) the antiserum was diluted 1 200,000 and the tubes were coated ith 10 ng per ml of LPS In the anti-B a./Y.e. LPS system (b) the corresponding figures were 1.50,000 and 5,000 ng per ml of LPS. Symbols of LPS used as inhibitors *B. abortus*, ● *Y enterocolitica* O-V × *Y enterocolitica* O-I ▼ *S typhi* T2, Δ

colitica O-V– *Y enterocolitica* O-V LPS no inhibition was seen if the highest concentration of *B abortus* LPS were used (1 000 μg/ml) The LPS from *Y enterocolitica* O-I was a powerful inhibitor but the amount for 50 per cent inhibition was four times higher than the amount of the homologous antigen.

The reaction between anti-*Y enterocolitica* O-I and tubes coated with *Y enterocolitica* O-I LPS was inhibited only with the homologous LPS. Surprisingly enough, the *Y enterocolitica* O-V did not inhibit in the highest concentration tested (1,000 μg/ml) *B. abortus* and *S typhi* T2 were not tested as inhibitors in this system.

In none of the systems did *S typhi* T2 LPS

TABLE 2 *Inhibition of ELISA with Different LPS Preparations Using Suboptimal Concentrations of Antisera against* B abortus, Y enterocolitica *O-V and O-I in Tubes Coated with Different LPS Species*

Inhibitory system, antibody/antigen	μg LPS per ml for 50% inhibition			
	B. abortus	*Y enterocolitica* O-V	*Y enterocolitica* O-I	*S typhi* T2
anti-*B abortus*/ *B abort* LPS	0.1	100	600	>1,000
anti-*B abortus*/ *Y enterocolitica* O-V LPS	0.003	3	30	>1,000
anti-*Y enterocolitica* O-V/ *B abortus* LPS	0.003	50	200	>1,000
anti-*Y enterocolitica* O-V/ *Y enterocolitica* O-V LPS	>1,000	5	20	>1,000
anti-*Y enterocolitica* O-I/ *Y enterocolitica* O-I LPS	ND*	>1,000	20	ND*

Not done

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O factor 9 antiserum (10) and RNS (10) against *Y enterocolitica* O I LPS The slope of the titration curve of *B abortus* antiserum however, differed significantly from the slopes of the other sera.

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Inhibition of ELISA with LPS

The capacity of the four LPS species to inhibit the reaction between anti-*B. abortus* and homologous or *Y enterocolitica* O-V LPS, anti *Y enterocolitica* O-V and homologous or *B abortus* LPS as well as anti *Y enterocolitica* O I and homologous LPS was tested using ELISA (Fig 3 Table 2)

B abortus LPS was a thousand times more efficient than *Y enterocolitica* O-V LPS to inhibit the binding of anti *B abortus* antibodies to tubes coated with homologous or *Y enterocolitica* O-V LPS (Fig 3) The inhibiting power of LPS from *Y enterocolitica* O I was from six to ten times less efficient than that of LPS from *Y enterocolitica* O-V When anti *Y enterocolitica* O V serum was used in tubes coated with *B.abortus* LPS there was an even greater difference in inhibition between the LPS preparations from *B abortus* and *Y enterocolitica* O-V *B. abortus* was about 17 000 times more powerful as inhibitor LPS from *Y enterocolitica* O-I but was about four times less efficient compared to *Y enterocolitica* O-V LPS.

In the homologous system anti-*Y entero-*

efficient compared to *Y enterocolitica* O-V LPS in all inhibitory systems involving anti-*B. abortus* serum and/or *B. abortus* LPS (Table 2). One possible explanation is that the number of cross-reacting determinants per molecule is greater on *B. abortus* LPS than on *Y enterocolitica* LPS. Another explanation is differences in solubility. The fact that *B abortus* LPS did not inhibit the anti-*Y enterocolitica* O-V–*Y enterocolitica* O-V LPS system (Table 2) probably means that antibodies against *Yersinia* specific antigens predominate in the serum dilution used. It applies also to the anti-*Y enterocolitica* O-I *Y enterocolitica* O-I LPS system that the lack of inhibition with *Y enterocolitica* O-V LPS could be ascribed to the antiserum dilution used. The weak, but apparently significant, inhibition with *Y enterocolitica* O-I LPS seen in the three inhibitory systems involving anti-*B. abortus* and/or *B abortus* LPS has not been observed earlier. It is difficult to find explanations other than common or cross-reacting determinants, especially since *S typhi* T2 LPS does not inhibit in any of the test systems tried.

Although based so far only on one observation, the introduction of ELISA in its present form may offer an interesting possibility for an effective immunochemical differential diagnosis of *Brucella abortus* and *Y enterocolitica* O-group V infections. Work is in progress with a view to having this observation verified and animal sera as well as human sera are used for this purpose. The ELISA test, so far involving tube agglutination, also demonstrates unknown cross-reactivities between *Y enterocolitica* O-group I and V LPS (Table 1) and between *B. abortus* and *Y enterocolitica* O-I (Table 2). The chemical nature of these cross-reactions is presently under study.

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REFERENCES

1. *Carlsson, H. E., Lindberg, A. A. & Hammarström, S.*: Titration of antibodies to *Salmonella* O-antigens by enzyme-linked immunosorbent assay. Infect. Immunity 6: 703–708, 1972.
2. *Carlsson, H. E., Lindberg, A. A., Hammarström, S. & Ljunggren, Å.*: Quantitation of *Salmonella* O-antibodies in human sera by enzyme-linked immunosorbent assay (ELISA). Int. Arch. Allergy *48*: 485–494, 1975.
3. *Engvall, E. & Perlmann, P.*: Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry *8*: 871–874, 1971.
4. *Engvall, E. & Perlmann, P.*: Enzyme-linked immunosorbent assay. III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen coated tubes. J. Immunol. *109*: 129–135, 1972.
5. *Engvall, E. & Ljungström, I.*: Detection of human antibodies to *Trichinella spiralis* by enzyme-linked immunosorbent assay, ELISA. Acta path. microbiol. scand. Sect. C *83*: 231–237, 1975.
6. *Hurvell, B.*: Serological cross-reactions between different *Brucella* species and *Yersinia enterocolitica*. Immunodiffusion and immunoelectrophoresis. Acta Vet. Scand. *13*: 472–483, 1972.
7. *Hurvell, B.*: Serological cross-reactions between different *Brucella* species and *Yersinia enterocolitica*. Agglutinating activity of 19 S and 7 S antibodies against somatic antigen of *Brucella abortus* and *Yersinia enterocolitica* type IX. Acta Vet. Scand. *14*: 1–15, 1973.
8. *Hurvell, B.*: Serological cross-reactions between different *Brucella* species and *Yersinia enterocolitica*. Biological and chemical investigations of lipopolysaccharides from *Brucella abortus* and *Yersinia enterocolitica* type IX. Acta path. microbiol. scand. Sect. B, *81*: 105–112, 1973.
9. *Hurvell, B. & Lindberg, A. A.*: Serological cross-reactions between different *Brucella* species and *Yersinia enterocolitica*. Immunochemical studies on phenol-water extracted lipopolysaccharides from *Brucella abortus* and *Yersinia enterocolitica* type IX. Acta path. microbiol. scand. Sect. B, *81*: 113–119, 1973.
10. *Knapp, W. & Thal, E.*: Die biochemische Charakterisierung von *Yersinia enterocolitica* (syn. *Pasteurella* X*) als Grundlage eines vereinfachten O-Antigenschemas. Zbl. Bakt., I. Abt. Orig. 223: 88–105, 1973.
11. *Lindberg, A. A. & Holme, T.*: Evaluation of some extraction methods for the preparation of bacterial lipopolysaccharides for structural

inhibit at the highest concentration tested (1 000 μg/ml) confirming the lack of cross-reactions between this species and *B abortus* and the two *Yersinia* species

DISCUSSION

Previous investigations using the enzyme-linked immunosorbent assay (ELISA) with extracted lipopolysaccharide (LPS) as antigen for the detection and quantitation of antibodies against the somatic O antigen of the cell envelope of *Salmonella* bacteria have shown it to be a sensitive and specific assay (1 2) It was recently established that the antigenic determinants used for serological diagnosis of bacteria belonging to the *Brucella* and *Yersinia* species were found in the LPS of the cell envelope (9) On the assumption that ELISA would be of equal importance for titration of antibodies formed against the *Brucella* and *Yersinia* species an experimental system was set up The results will be discussed, focusing on the sensitivity and specificity of the test, with special emphasis on the possibility of a differential diagnosis between *Brucella abortus* and *Yersinia enterocolitica* O-V (formerly designated type O 9)

When the titre in ELISA was compared to the titre observed with the tube agglutination method it was regularly observed that the ELISA was from 10 to more than 100 fold more sensitive (Table 1) This is in accord with the results obtained in the determination of antibody titres against the O-antigens of *Salmonella* bacteria (1 2) The largest differences were found in the titres of the pooled hyperimmune sera against *Brucella abortus* and *Yersinia enterocolitica* O-V When the antisera were separated by gelfiltration on Sephadex G-200 (data not shown) into IgM and IgG containing fractions, and subsequently tested in ELISA, more than 90 per cent of the total antibody activity was found in the IgG containing fractions. This partly explains the large difference between the tube agglutination and ELISA titre determinations with these hyperimmune sera. ELISA detects IgG and IgM antibodies with about equal sensitivity whereas the tube agglutination test preferentially detects IgM antibodies (1)

One interesting observation in the coating efficiency of the different LPS preparations was observed The amount of LPS from *Brucella abortus* for optimal coating was found to be 10 ng/ml, whereas the corresponding amounts for LPS from the two *Y enterocolitica* and the *S typhi* strains were 5 000 ng/ml, a fivehundredfold difference. One possible explanation is a difference in solubility of the LPS preparations. Chemical analyses have shown that the *B. abortus* LPS contains relatively more lipid than the other LPS preparations used and that there are qualitative differences in respect of the fatty acid composition (*Stenhagen* personal communication) Besides differences in solubility this could also affect the binding of the LPS to the plastic tube which takes place without any coupling agent, presumably through hydrophobic interaction.

The observed serological cross-reaction between the different *Brucella* species and *Y enterocolitica* O-group V seriously interferes with the reliability of the immunological differential diagnosis between strains belonging to these genera The ELISA, besides being more sensitive also raises the possibility of an improved immunochemical differential diagnosis When the tubes were coated with the *B abortus* LPS as antigen and the pooled hyperimmune sera against *B. abortus* and *Y enterocolitica* O-V were tested, the homologous titre was 100-fold higher than the heterologous 300 000 and 3 000 respectively (Fig 2a and Table 1) In tube agglutination tests the corresponding difference was only four fold (Table 1)

A similar difference could however not be observed when the *Y enterocolitica* O-V LPS was used as antigen for coating of the tubes (Fig 2b and Table 1)

Inhibition studies confirm the cross-reactions observed in antibody titration experiments. A remarkable observation is, however that *B abortus* LPS is thousandfold or more

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THE EFFECT OF ANTI LYMPHOCYTE SERUM, MITOGENS AND ENZYMATIC TREATMENT ON THE AGGLUTINATION AND SURFACE CHARGE OF LYMPHOID CELLS

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Wiig, J N The effect of anti-lymphocyte serum, mitogens and enzymatic treatment on the agglutination and surface charge of lymphoid cells. Acta path. microbiol. scand. Sect. C, 84 177–184 1976

Agglutination of thymocytes and B-cells with anti-lymphocyte serum subsequent to treatment of the cells with trypsin or neuraminidase was slightly and similarly increased, independent of the effect on the surface charge shown previously The enzymatic treatment did not increase capping of antiserum binding sites. Agglutination caused by binding of IgG antibodies of anti-lymphocyte serum was not accompanied by change in the net surface charge. Neither did binding of the mitogens phytohaemagglutinin, concanavalin A or poke-weed mitogen influence the surface charge of the cells

Key words Lymphoid cells agglutination surface charge ALS mitogens enzymes.

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Cell membrane structures of lymphoid cells are of fundamental importance for the initiation of the immune response (8) Another property of these membranes is a net negative surface charge (2, 4 6 15 26, 31 32) Reduction of this, and thereby a reduction in the intercellular repulsive forces, has been suggested to be of importance for the agglutination or aggregation of erythrocytes (18) lymphoid cells (5 6) or thrombocytes (9) as well as the mitogenic stimulation (24)

Several workers have found that binding of anti-lymphocyte sera (ALS) (2, 3 4 17) to lymphoid cells causes a reduction in the net negative surface charge of the cells, while the effect of mitogens on the surface charge seems in part contradictory (5 6 13 24 30) Most of these studies were not concerned with the possible influence of a capped distribution of the reactants on the cell surface.

Similarly reduction in net surface charge caused by enzymatic treatment of erythrocytes has been held responsible for the subsequent increase in agglutination titre of sera (18) and the facilitated demonstration of incomplete antibodies. Recently it has been suggested in studies on fibroblasts that the

analysis. Acta path. microbiol scand. Sect. B *80* 751–759 1972
Westphal O., Lüderitz O & Bister F Über die Extraktion von Bakterien mit Phenol Wasser Z. Naturforsch. *7* 148–155 1952.
13 *Winbladh S.*, Studies on serological typing of *Yersinia enterocolitica* Acta path. microbiol. scand., Suppl *187* 115 1973

11 Acta path. microbiol. scand. Sect. C, 84: 177–184, 1976

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Key words: Lymphoid cells; agglutination; surface charge; ALS; mitogens; enzymes.

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Cell membrane structures of lymphoid cells are of fundamental importance for the initiation of the immune response (8). Another property of these membranes is a net negative surface charge (2, 4, 6, 15, 26, 31, 32). Reduction of this, and thereby a reduction in the intercellular repulsive forces, has been suggested to be of importance for the agglutination or aggregation of erythrocytes (18), lymphoid cells (5, 6) or thrombocytes (9) as well as the mitogenic stimulation (24).

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Similarly, reduction in net surface charge caused by enzymatic treatment of erythrocytes has been held responsible for the subsequent increase in agglutination titre of sera (18) and the facilitated demonstration of incomplete antibodies. Recently it has been suggested in studies on fibroblasts that the

enzymatically induced increase in agglutination titre might be due to aggregation of agglutinating sites on the cell surface (14)

In the present study the general importance of the surface charge and capping for cell agglutination was examined The effect of trypsin neuraminidase and phospholipase C treatment of lymphoid cells on the agglutinating titre of ALS was tested and the results were evaluated in the light of previously shown effects on the net surface charge of the cells (27 28) The influence of the enzymatic treatment on the surface distribution of ALS was studied by immunofluorescence. The net surface charge as measured by the electrophoretic mobility (EPM) of murine lymphoid cells was tested with agglutinating ALS and the mitogens phytohaemagglutinin (PHA) concanavalin A (con A) (20) and poke weed mitogen (PWM) (7)

MATERIALS AND METHODS

Mice C3H/A/BOM male mice 2–4 months old from Gamle Bomholtgaard Laven, Denmark were used

Reagents Neuraminidase was obtained from Behringwerke AG Marburg am Lahn, Germany trypsin (T 8253) deoxyribonuclease (I DN 100) phospholipase C (L, P 7633) and Con A from Sigma Chemical Corporation St. Louis, U.S.A PHA from Wellcome Research Laboratories, Beckenham, England and PWM from Grand Island Biochemical Corporation, New Jersey U.S.A. The effect of these enzyme batches had been tested previously (27 28)

Antisera Anti-lymphocyte sera were prepared in rabbits against lymph node cells (ALS I) and thymocytes (ALS II ALS III) as described by *Jooste et al* (10) The sera were heat inactivated (56 C, 30 min) ALS I was absorbed twice with an equal volume of packed and washed mouse erythrocytes overnight. This serum was also treated with mercapto-ethanol (ME) (11) By immunoelectrophoresis similar treatment was shown to destroy IgM antibodies of human serum Some activities of the sera and the ME resistant fraction of ALS I is given in Table 1

Goat anti rabbit immunoglobulin serum was prepared. The antiglobulin fraction was conjugated with fluorescein isothiocyanate (FITC) for the immunofluorescence tests (21) and used unconjugated in the sandwich technique for EPM studies.

Cells. Thymocytes and lymph node cells were obtained from normal mice T-cell spleens by cyclophosphamide ("Sendoxan" Pharmacia, Sweden) treatment (22 26) and B-cell spleens by adult thymectomy irradiation and bone marrow reconstitution (26) Cell suspensions were prepared as described previously (25 29) Viability of the cell suspensions was 85–95 per cent as estimated by trypan blue exclusion.

Enzymatic treatment The enzymes were prepared in Hanks balanced salt solution (HBSS) with 0 005 M $CaCl_2$. Trypsin was used at a final concentration of 0 125 mg/ml with added 0 1 mg deoxyribonuclease phospholipase C at a slightly lytic concentration of 0 6 µg/ml and neuraminidase at 100 I U./ml The cells were resuspended in the enzyme solutions to a concentration of 100 x 10^6 cell/ml and incubated at 37 C for 30 min except for the neuraminidase treatment where pH was adjusted to 7.2 and the incubation performed at 37 C for 60 min.

Agglutination tests were performed by incubating 10 µl cells (10^7 cells/ml HBSS) and 10 µl reactant solution at 37 C for 30 min and reading in a Bürker counting chamber after gentle resuspension of the cells. The agglutination was evaluated as + ++ or +++ corresponding to 25 50 and 75 per cent agglutination of cells. Doubling dilutions up to 1 1024 were tested ALS and the ME resistant fraction were used in ten twofold dilutions from 1 in 4 An indirect Coombs test was performed with antiglobulin immunoglobulin serum diluted 1 50 to check whether the ME resistant antibodies might be of the IgG class. PHA and PWM were prepared 1 in 10 and in five twofold dilutions from 1 in 100 and Con A in four tenfold dilutions from 1 mg/ml The tests were read blind.

Cytotoxic testing with ALS was performed with guinea pig serum absorbed with agar diluted 1 in 12 in HBSS as complement source. Equal volumes (10 µl) of serum, complement and cell suspension (10^7 cells/ml HBSS) were incubated at 37 C for 60 min Then 10 µl 0.2 per cent trypan blue was added and the percentage of dye excluding cells was read in a Bürker counting chamber after incubation for 30 min at 20 C. The antisera were used in ten twofold dilutions from 1 in 4

Immunofluorescence tests A sandwich technique using ALS (dilution 1 in 8 for surface distribution test) and antiglobulin (dilution 1 in 2) was performed (24) To safeguard that distribution was even, it was tested by incubation at 20° C with both antisera or at 37 C with ALS and at 4 C with antiglobulin The cells were washed with HBSS containing 0 03 M sodium azide at 20 C or 4 C Capped distribution was studied after incubation at 37 C with both antisera and washing with HBSS after first incubation and with HBSS containing sodium azide after the second.

Treatment of cells for EPM studies ALS were used at agglutinating titres (I 1 in 8 II 1 in 128, III 1 in 256) and antiglobulin 1 in 2 for the sand

TABLE 1 *Titres of Different ALS, Mercaptoethanol (ME) Treated ALS I and ALS I/ME Incubated with Goat Anti-rabbit Immunoglobulin G Serum (Antiglobulin) against Thymocytes (TC) or Whole Lymph Node (LN) Cells*

Antiserum	Agglutination		Cytotoxicity‡	Immunofluorescence
	TC	LN	LN	TC
ALS I	16	16	256	32
ALS II	256	256	256	32
ALS III	512	512	1024	64
ALS I/ME	16			
ALS I/ME/antiglobulin	64			

‡ 50 per cent killing as end point.
The titres are expressed as reciprocals of the highest dilutions at which a positive reaction could be observed

wich technique. All incubations were performed for 30 min. Three series of experiments were carried out to test the effect of ALS treatment of EPM. Firstly the effect of ALS I, II and III on T-spleen cells incubated at 20° C was studied. Secondly the influence of the surface distribution of antibodies on thymocytes with ALS I was studied using sandwich technique. To safeguard that distribution was even, it was tested by incubation at 37° C with ALS I alone while capping was achieved by a subsequent incubation in antiglobulin at 37° C as described above. Thirdly thymocytes were suspended in physiological saline subsequent to incubation with ALS I at 20° C to test the effect of low conductivity suspending medium as used in the foregoing experiments.

The mitogens were used at the following concentrations: PHA at 1 in 500 (higher concentrations caused extensive agglutination during washing in electrophoresis medium), Con A at 10 µg/ml which gives optimal binding (16) and PWM at 1 in 100 (as advised by the manufacturer). Thymocytes were tested with all three mitogens and B-spleen cells with PWM. Incubations were performed for 30 min at 4° C or 37° C to test the effect on EPM of even or capped distribution of the reactants (12, 23).

Cell electrophoresis was performed as previously described (25, 27). The cell incubated at 4° C or 37° C were suspended in a medium of relatively low ionic strength consisting of 5.25 per cent glucose and 0.03 M sodium azide buffered with 0.03 M Tris, pH 7.2. Cells incubated at 20° C were suspended in glucose medium without sodium azide. In some experiments with ALS, the cells were suspended in physiological saline. Only morphologically viable, small cells (<8 µm) were scored. The EPM is given as µm/sec/V/cm ± standard deviation.

Statistical methods. The mean EPM of lymphoid cells was recorded relative to a mean EPM of human reference erythrocytes of 1.6470 for glucose/sodium azide medium, 1.6551 for cells scored in glucose medium (27) and 1.2500 for physiological saline (25). Frequency polygons were drawn. The statistical significance of differences between groups of cells was calculated by the Student's t-test.

RESULTS

Agglutination Studies

The agglutination titres of the tested sera are given in Tables 1 and 2. The activity of ALS I was not reduced by ME-treatment while the agglutinating activity of the ME resistant fraction was increased by two doubling dilution steps subsequent to a second incubation in goat anti rabbit immunoglobulin serum.

Following treatment of thymocytes and B-spleen cells with trypsin or neuraminidase, the agglutination titre of ALS I was increased from that of simultaneous controls by one or two doubling dilution steps in four and three experiments, respectively. Total agglutination score was increased from 5 to 8–11. Treatment of thymocytes with phospholipase C did not alter their agglutinability (Table 2). No agglutination with any of the mitogens in the concentrations studied was found. However unlike the other mitogens, PHA in dilutions up to 1 in 250 aggregated the cells extensively during subsequent centrifugation in glucose-medium.

TABLE 2 *The Effect of Enzymatic Treatment of Thymocytes and B-spleen Cells (See Text) on the Agglutination of ALS I*

Source of cells	Enzyme	ALS dilution§						
		1/4	1/8	1/16	1/32	1/64	1/128	Total score
Thymus	Nil	+ +	+ +	+	–	–	–	5+
	Trypsin	+ + +	+ + +	+ +	+ (+)*	(+)	–	10+
	Neuraminidase	+ +	+ +	+ +	+ (+)	(+)	–	8+
	Phospholipase C	+ +	+ +	+	–	–	–	5+
B-spleen	Nil	+ +	+ +	+	–	–	–	5+
	Trypsin	+ + +	+ + +	+ +	+ +	+	–	11+
	Neuraminidase	+ + +	+ +	+ +	+ (+)	(+)	–	9+

§ + = 25 per cent agglutinates.
+ + = 50 per cent agglutinates.
+ + + = 75 per cent agglutinates
* ( ) = score in 3/7 of experiments.

Fluorescence Studies

ALS was evenly distributed on the surface of thymocytes, i.e. 87 per cent positive cells and only 8 per cent caps (Table 3) after performance of both incubations at 20° C using the sandwich technique Similar results were found when incubation with ALS I at 37 C was followed by incubation with anti globulin at 4° C In contrast 30 per cent caps were obtained the total number of fluorescent cells not changing when both incuba tions were performed at 37° C

No definite effect of trypsin, neuraminidase or phospholipase C on the capping phenomenon was found

Electrophoresis Studies

Control cells The EPM of T-spleen cells suspended in glucose medium without sodium azide was 1 61 ± 0.20 (Table 4) that of thymocytes in glucose medium with added sodium azide being 1 28 ± 0 19 (Table 5) and in physiological saline 0 96 ± 0 18 (Table 5) B-spleen cells in glucose/sodium azide medium had an EPM of 1 19 ± 0.25 (Table 6)

ALS incubation Incubation of T-spleen

TABLE 3 *The Effect of Enzymatic Treatment of Thymocytes on Capping after Subsequent Incubation of Thymocytes with ALS I and Antiglobulin*

Enzyme	Temperature (C) at incubation with		Per cent fluorescent cells	
	ALS	antiglobulin	Total	Capping*
Nil	20	20	87	8
Trypsin	20	20	88	16
Neuraminidase	20	20	96	6
Nil	37	4	95	3
Trypsin	37	4	85	15
Neuraminidase	37	4	96	6
Phospholipase C	37	4	92	3
Nil	37	37	86	30
Trypsin	37	37	92	35

* Capping is given in per cent of total number of fluorescent cells.

TABLE 4 *The Mean Electrophoretic Mobility (EPM) in Glucose Medium of T-spleen Cells Incubated in Vitro at 20° C in Different ALS*

Antiserum	EPM	Δ	p <
Nil	1.61 ± 0.20 (103)		
ALS I	1.66 ± 0.20 (41)	+3	0.20
ALS II	1.71 ± 0.13 (30)	+6	0.001
ALS III	1.61 ± 0.20 (30)	0	

EPM is given as μm/sec/V/cm ± standard deviation. Number of cells scored in parenthesis.
Δ Per cent change

TABLE 5 *The Mean Electrophoretic Mobility (EPM) in Glucose/Sodium Azide Medium of Thymocytes Incubated in Vitro at 37° C in ALS I either Alone or in Sandwich Technique with Antiglobulin at 37° C or in Physiological Saline Subsequent to Incubation in ALS I at 20° C*

EPM medium	Antiserum	EPM	Δ	p <
Glucose/sodium azide	Nil	1.28 ± 0.19 (250)		
	ALS I	1.27 ± 0.28 (402)	−1	0.60
	ALS I/antiglobulin	1.26 ± 0.23 (141)	−2	0.40
NaCl	Nil	0.96 ± 0.18 (150)		
	ALS I	0.89 ± 0.17 (186)	−7	0.001

EPM is given as μm/sec/V/cm ± standard deviation. Number of cells scored in parenthesis.
Δ Per cent change

TABLE 6 *The Mean Electrophoretic Mobility (EPM) in Glucose/Sodium Azide Medium of Thymocytes and B-spleen Cells Incubated in Vitro in PHA 1:500 Con A 10 μg/ml or PWM 1:100*

Source of cells	Temperature of incubation (° C)		EPM	Δ	p <
Thymus	Nil		1.28 ± 0.19 (250)		
	PHA	4	1.22 ± 0.23 (86)	−5	0.05
	PHA	37	1.24 ± 0.27 (150)	−3	0.20
	Con A	4	1.25 ± 0.18 (200)	−2	0.10
	Con A	37	1.25 ± 0.21 (190)	−2	0.10
	PWM	4	1.21 ± 0.20 (100)	−5	0.005
	PWM	37	1.25 ± 0.22 (100)	−2	0.30
B-spleen cells	Nil		1.19 ± 0.25 (84)		
	PWM	4	1.21 ± 0.27 (129)	+2	0.70
	PWM	37	1.19 ± 0.30 (173)	0	

EPM is given as μm/sec/V/cm ± standard deviation. Number of cells scored in parenthesis.
Δ Per cent change

cells with different ALS increased the EPM by 0–6 per cent (Table 4). Even distribution of ALS I alone on the surface of thymocytes, or at capped conditions with antiglobulin reduced the EPM by 1–7 per cent, irrespective of electrophoresis medium (Table 5). All these figures are within the error of measurement although some statistically were highly significant. No new sub-groups were found on the frequency polygons.

Mitogen incubation The individual data are given in Table 6 The mean EPM of thymocytes incubated with any of the three mitogens at 4 C or 37° C was 2–5 per cent lower than that of control cells. The EPM of B-spleen cells incubated with PWM was increased by 2 per cent These figures are within the error of measurement Frequency polygons did not show any new sub-grouping

DISCUSSION

As apparent from the lacking ability of incomplete antibodies to cause cell agglutination under ordinary conditions, factors other than the mere binding of antibodies may be involved in the agglutination process (18) Studies of erythrocytes have shown that conditions favouring agglutination by incomplete antibodies also caused reduction in the net negative surface charge of the cells (18) In addition the surface charge was found to be reduced after binding of agglutinating antibodies to the cell surface. Accordingly such reduction of the net surface charge and thus of the intercellular repulsive forces, was considered an important factor in the cell agglutination process. The reduction might be due to a splitting off of charged groups to or a binding of substances to the cell membrane. Bound molecules might add to or neutralize charges on the cell membrane, or cause considerable re-orientation of groups e g "capping" endocytosis or "shedding"

In the present experiments the increase in agglutinability subsequent to treatment of the cells with trypsin or neuraminidase was found to be consistent. Although slight it is therefore considered real. This increase in agglutinability was not accompanied by an increased capping of the bound ALS On the assumption that capping induced agglutination might have taken place at lower concentrations of antibody this would have appeared as a second agglutination peak" which was not found at dilutions up to 1 1024 This is in contrast to findings in studies of enzyme induced con A agglutination of fibroblasts (14) It has been shown previously (28) that similar trypsin treatment will reduce the net surface charge of thymocytes by 10 per cent with no definite effect on the B-cell charge, while neuraminidase will reduce the surface charge of both cell types by about 40 per cent (27) Thus the present studies indicate that the increased agglutinability caused by the enzymatic treatment cannot be explained either by an increased aggregation of agglutinating sites or by a reduction in the net surface charge and thus by the repulsive forces between cells. Under the present conditions, trypsin, in contrast to neuraminidase, does not seem to split off sialic acid molecules from the membrane of lymphoid cells (27 28) Thus, the increased agglutination caused by the enzyme treatment might be due to an uncovering of increased number of antigenic groups reacting with the anti lymphocyte antibodies in the ALS or of new groups of antigens reacting with naturally occurring antibodies in the antiserum (19) The increased agglutination is unrelated to phospholipid groups susceptible to phospholipase C.

The second incubation in the sandwich technique at 4 C will not change the surface distribution of the primarily bound antibodies (23) As shown in the immunofluorescence studies, incubation of thymocytes with ALS alone gave an even distribution of the antibodies on the cell surface. The lack of effect of ME treatment of ALS I and the increased titre in the indirect Coombs test indicated that the agglutinating effect was due to antibodies of the IgG class. Thus, under these conditions the present study failed to show that antibodies of the IgG class bound to thymocytes or T-cells had any effect on the net surface charge of the cells in high or low conductivity media. This is in contrast to previous studies using ALS (2 3 4 17) which however do not state the class of the relevant antibodies. While binding of antibodies at the dilution used could be shown by agglutination as well as by cytotoxic and immunofluorescent tests, the present study indicates that the EPM technique cannot be considered a test that is useful in general for cell bound antibodies of the IgG class.

Binding of the mitogens PHA and con A to thymocytes and of PWM to B-cells and thymocytes did not affect the EPM. Lack of effect of PHA is in accordance with a previous report on lymphoid cells (13) but in contrast to three others (5 6, 24) In one study of con A (30) and in one of PWM (13) a lack of effect of these mitogens on the EPM is also reported.

Capping was shown in about one third of the thymocytes after incubation with ALS and antiglobulin by the sandwich technique at 37 C. Capping as well as endocytosis could be expected to take place during incubation with the mitogens, at least PHA (1 12) and con A (23) at the same temperature. Nevertheless no definite change in the EPM of the frequency polygons was found. The lack of effect of the capping phenomenon on EPM by these reagents is in contrast to a recent report according to which 11 per cent increase in the mean EPM was found after con A induced capping and endocytosis (30)

Although most of the EPM studies were performed in medias of low conductivity and *the agglutination titrations in high conductivity medias*, several points suggest that valid conclusions concerning the correlation between surface charge and agglutination can be drawn. Firstly one of the antisera failed to change the EPM whether tested in low or high conductivity medias. Secondly it seems unlikely that an ALS induced change in surface charge could be neutralized by suspending the cells in low conductivity medium. Thirdly any significant dissolution of ALS molecules bound at the cell membrane seems unlikely as incubated cells during the washing procedure *in low conductivity medium* showed a tendency to agglutination at least as great as that of cells washed in medium of high conductivity

The present study thus indicates that, although the net negative surface charge may play a part in the mechanism of cell agglutination, as shown on erythrocytes (18) it does not seem to be a major factor under the conditions used in the present study which probably are similar to those used in most laboratories. Furthermore, neither a reduction in the net surface charge, nor an increased clustering of agglutinating sites can be accepted as general explanations of the increased agglutinability of cells *after treatment* with some enzymes.

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REFERENCES

1 *Adler W H., Osunkoya, B. O Takiguchi T & Smith R. T* The interactions of mitogens with lymphoid cells and the effect of neuraminidase on the cell responsiveness to stimulation. Cell. Immunol. *3* 590–605 1972.

2 *Bert G Cassano D L., Pecco P & Mazzei D* Effect of ALS and prednisolone on the electrophoretic mobility of lymphocytes. *Lancet i* 365 1970

3 *Bert G Ferrettar J A & Davies A J A* Differential response of B- and T-cells to ALS revealed by electrophoretic mobility determinations. N ture (New Biol) *234* 86–87 1971

4 *Bona C Anteunis A. Robineaux R & Halpern B* Structure of the lymphocyte membrane III Chemical nature of the guinea-pig lymphocyte membrane molecules reacting with heterologous ALS. Clin. exp. Immunol *12* 377–390, 1972.

5 *Caspary E. A & Knowles M* Effect of haemagglutinating mitogenic fractions of phytohaemagglutinin on electrophoretic mobility of lymphocytes and macrophages Separatum Experientia *26* 1238–1239, 1970.

6. *Currie G A* Effect of phytohaemagglutinin on the surface charge of human and murine lymphocytes Nature *216* 694–695 1967

7 *Greaves M & Janossy G* Elicitation of selective T and B-lymphocyte responses by cell surface binding ligands. Transpl. Rev *11*: 87 130 1972.

8. *Greaves M F Owen J J T & Raff M C.* T and B lymphocytes. Excerpta Medica American Elsevier Amsterdam 1973 p. 113–148.

9 *Grottum K A* Platelet surface charge and aggregation. Effects of polyelectrolytes. Thromb. Diathes. haemorrh. *21* 450–462, 1969

10 *Jeeds S V Lance E M Levey R. H Medawar P B., Ruszkiewicz M Sharman R. & Taub R. N* Notes on preparation and

assay of antilymphocytic serum for use in mice. Immunology *15* 697–705 1968

11 *Kabat E. A & Mayer M M* Experimental Immunochemistry 2ed. C. C. Thomas Publisher Springfield USA, 1961 p 351

12. *Loor F* Lymphocyte membrane particle redistribution induced by a mitogenic/capping dose of the phytohemagglutinin of *Phaseolus* vulgaris. Eur J Immunol. *3* 112–116 1973

13 *Mitchell D M & Cater D B* The electrophoretic mobility of BP8 ascites tumour cells and allergized lymph-node cells after treatment with inflammatory mediators, ptomaines, polyamines, antisera and neuraminidase or heparin. Brit J exp Path. *52* 152–171 1971

14 *Nicolson G L* Temperature-dependent mobility of concanavalin A sites on tumour cell surface. Nature (New Biol) *243* 218–220 1973

15 *Nordling S Anderson L C & Häyry P* Separation of T and B lymphocytes by preparative cell electrophoresis. Eur J Immunol 2 405–410 1972

16 *Parker C W Snider D E & Wedner H J* The role of cyclic nucleotides in lymphocyte activation In Brent, L. & Holborow J (Eds.) Progress in Immunology II 2 North Holland, Amsterdam, 1974 p 85–94

17 *Phondke G P Sundaram K & Sundaresan P* Effect of antilymphocyte serum on the electrophoretic mobility of the lymph node cells of the rat. Nature *225* 79–80 1970

18 *Pollack W* Some physicochemical aspects of hemagglutination. An New York Acad. Sci. *127* 892–900 1968.

19 *Ray P K Gewurz H & Simmons R L* The serologic behaviour of neuraminidase-treated lymphoid cells. Alloantigenicity and complement sensitivity Clin. exp Immunol *11* 441–460 1972

20 *Stobo J D* Phytohemagglutinin and concanavalin A Probes for murine T cell activation and differentiation Transpl Rev *11* 60–86 1972

21 *Thunold S Horsdal K & Hartveit F* Complement factors and the growth of Ehrlich's carcinoma. Acta path microbiol scand Sect. B, *81* 373–381 1972

22 *Turk J L & Poulter L W* Selective depletion of lymphoid tissue by cyclophosphamide Clin. exp. Immunol *10* 285–296, 1972

23 *Unanue E R Perkins W D & Karnovsky M J* Ligand induced movement of lymphocyte membrane macromolecules I Analysis by immunofluorescence and ultrastructural radiography J exp. Med. *136* 885–906 1972

24 *Lasser P S & Culling C F A* Cell surface effects of phytohaemagglutinin. Nature *202* 610–611 1964

25 *Wug J N* Electrophoresis of lymphoid cells. Organ specific distribution pattern of small lymphoid cells in the mouse. Scand. J Immunol *2* 23–29 1973

26 *Wug J N* Electrophoresis of lymphoid cells Characterization of T and B-lymphocytes and two populations of thymocytes in the mouse. *Acta path. microbiol. scand. Sect. A, Suppl 236* 101–111 1973

27 *Wug J N* Effect of neuraminidase on lymphoid cells. Difference in structure of B- and T-cells and thymocytes of the mouse shown by cell electrophoresis and sialic acid determination. Scand J Immunol. *3* 357 363 1974

28. *Wüg J K* Electrophoresis of lymphoid cells. Differences in the cell membrane structure of murine thymocytes, T and B-cells revealed by enzyme and formalin treatment. Clin. exp. Immunol *22* 91–101 1975

29 *Wüg J N & Thunold S* Electrophoresis of lymphoid cells. Characterization of human B- and T-cells in peripheral lymphoid tissues. Clin exp Immunol. *15* 497–506 1973

30 *Wioland M* Electrophoretic mobility of the thymocyte during endocytosis of concanavalin A. Cell Immunol. *12* 472–475 1974

31 *Wioland M Sabolovic S & Burg C* Electrophoretic mobilities of T and B-cells. Nature (New Biol) *237* 274–276, 1972

32 *Zeiller K Holzberg E Pascher G & Hannig K* Free flow electrophoretic separation of T and B lymphocytes. Evidence for various subpopulations of B cells. Hoppe-Seyler's Z Physiol. Chem. *353* 105–110 1972.

Acta path. microbiol. scand. Sect. C, 84: 185–195, 1976.

DIFFERENTIAL EFFECT OF CORTISOL ON THYMOCYTES FROM MICE OF DIFFERENT AGES

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The influence of cortisol *in vivo* and *in vitro* was studied on thymus sections, suspensions, and smears obtained from new-born, 6 days old, and 30 days old mice. Histology as well as differential counts revealed that new-born thymocytes are far more sensitive to the cytotoxic effect of cortisol than thymocytes from older animals, and thymocytes from 30 days old mice are more sensitive than cells from 6 days old animals. Thus, the number of pyknotic cells in the thymus of new-born and 30 days old mice increased up to 20 per cent 6 h after injection of cortisol compared to 10 per cent in 6 days old animals. At 24 h after injection, the per cent of pyknotic cells in the thymus of new-born mice was 50 compared with about 1 in the two older age-groups. Supravital dye exclusion showed that thymocytes in 6 days old mice are less sensitive to the cytolytic action of cortisol *in vitro* than thymocytes from new-born and 30 days old mice. Incorporation of ^{3}H-TdR and ^{3}H-UdR into thymocyte DNA and RNA, respectively, was inhibited in all groups after treatment *in vivo*. DNA-synthesis of thymocytes in new-born animals was inhibited more than DNA-synthesis in thymocytes of older animals, whereas RNA-synthesis was inhibited to nearly the same extend in the three groups studied. Incubation *in vitro* with 10^{-6}M cortisol inhibited the DNA-synthesis to the same extent in thymocytes from the three age groups studied whereas the inhibiting effect of 10^{-6}M cortisol on thymocytes from 6 and 30 days old animals was more intense than that on cells in new-born mice. The RNA-synthesis of thymocytes from 6 days old mice incubated in 10^{-6}M cortisol was inhibited far less than RNA-synthesis in cells from new-born and 30 days old mice. The experiments reported, showing differential effects of cortisol on thymocytes obtained from mice of different ages, may reflect different stages of thymocytes maturation during the process of ontogenesis.

Key words: Cortisol; thymocytes; differential effect; mice.

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Treatment with corticosteroids results in involution of the lymphoid organs, the effect being most pronounced in the thymus. The involution is due to pyknosis, karyorrhexis, and cell lysis of small lymphocytes as well as inhibition of DNA-synthesis of lymphoid blast cells (3, 7, 9, 11, 13, 15, 16, 21).

However, thymic lymphocytes are not a homogenous group of cells but differ markedly in sensitivity to corticosteroids, the vast majority (above 90 per cent) being highly

sensitive to the cytotoxic actions of the hormones. Less than 10 per cent of the thymic lymphocytes are more resistant to corticoids and these are the cells which probably exhibit their immunological functions in peripheral lymphoid organs after having left the thymus by emigration (1, 6 10 20)

It has been demonstrated recently that thymic lymphoid cells obtained from mice of different ages differ with respect to responsiveness to mitogens and allogenic cells in vitro (4 17) and capability to elicit syngenic and allogenic mixed lymphocyte reactions (2) The age dependent differences in thymocyte reactivity are especially marked during the neonatal period. From the age of one month and onward only minor fluctuations in thymocyte reactivity are observed In the light of these studies we have examined the effects of cortisol *in vivo* and *in vitro* upon thymic lymphoid cells obtained from mice of three different ages, namely new born, six days old and thirty days old animals. Thymic weights percentage thymic lymphoid cell distribution including pyknotic cells and non viable cells examined by supravital dye exclusion technique, incorporation of ^{3}H thymidine (^{3}H TdR) and ^{3}H uridine (^{3}H UdR) were studied 6 and 24 h after one single injection of cortisol Furthermore the effects of cortisol *in vitro* on thymocyte incorporation of ^{3}H TdR and ^{3}H UdR were examined

MATERIAL AND METHODS

Animals New-born (6–12 h old) six days old and thirty days old female Balb/C mice from an inbred strain were used. They were housed under standard conditions, the light being turned on and off at 6 a.m. and 6 p.m respectively

In vivo *studies* The experimental animals were injected i p with 50 μg cortisol (hydrocortisone acetate dissolved in sterile 0 9 saline) per g body weight Control animals were injected with saline only Animals were sacrified by cervical dislocation 6 and 24 h after injection and thymuses removed weighted, and single-cell suspension made from one of the lobes in RPMI 1640 medium Nigrosin dye exclusion, smearing fixation and staining were performed at this stage as described previously (5) The other thymus lobe was fixed in neutral formaline and subjected to conventional paraffin technique. Sections were stained with HE

Counts Two thousand consecutive cells from each thymus smear were registered at 1250 x magnification. The cells were scored by an ocular grid in cells with nuclear diameter <8 μ (small thymocytes) and cells with nuclear diameter ≥ 8 μ (medium and large thymocytes) The pyknotic cells showed a dense homogenous nucleus being <6 μ in diameter

Incorporation of ^{3}H TdR and ^{3}H UdR. The initial cell suspension was washed once in RPMI 1640 and resuspended in this medium. Aliquots of 250 μl cell suspension were cultured for 1 h in tissue culture plates (3041 Falcon Plastics) at 38 C in humidified atmospheric air containing 5 per cent CO_2. Twenty μl ^{3}H TdR = 0.5 μC (N E.N specific activity 22 C/mMol) or 20 μl ^{3}H UdR = 2.0 μC (N E.N specific activity 20 C/mMol) were added to each culture. The cultures were harvested on filtres using a multiple Cell-Culture Harvester (Skatron Norway) Activity was counted in dioxan in a Beckmann LS-100C scintillation counter In some instances the cultured cells were harvested washed smeared, and prepared for autoradiography as described elsewhere (5)

In vitro *studies* Thymus cell suspensions were prepared as described above Cells were pooled from groups of mice according to age of the animals and incubated for from 2 to 6 h in ^{3}H TdR and ^{3}H UdR as described above At the beginning of the culture period 10^{-8} and 10^{-4} Mol cortisol was added to each culture At the termination of incubation cells were harvested and counted as mentioned above In some instances 0 2 per cent nigrosin was added to the 6 h cultures and the total number of viable cells determined

The experiments measuring incorporation of ^{3}H TdR and ^{3}H UdR after treatment with cortisol *in vivo* and *in vitro* were run in parallel with respect to time and age groups under study Thus, the inevitable day to day variations, which otherwise would make interpreparations impossible were avoided and direct comparison between the individual age groups could be performed.

RESULTS

Thymus weight Table 1 shows the thymus weights of mice killed 6 and 24 h after administration of cortisol During the first 6 h, the thymus weights were unaffected by treatment indicating that thymic cell numbers are kept at a rather constant level during this early period. Twenty four h after injection the weights had decreased with approximate-

TABLE 1 *Weights of Thymuses from Mic of Different Ages Tr at d with Saline (Controls) or Cortisol The Values of S.E.M A e R orded In the Table Each Value R presents the Mean of 3–6 Animals*

m after rection	New-born Control (A) mg	Treated (B) mg	Decrease (B/A)	Six days old Control (A) mg	Treated (B) mg	Decrease (B/A)	Thirty days old Control (A) mg	Treated (B) mg	Decrease (B/A)
6 h	4 4	4.3	0.97	18 2 ± 1.3	17.2 ± 0 4	0.94	80.9 ± 3.0	76.5 ± 4 4	0.95
24 h	6 4	3.0	0.47	23 3 ± 0 8	15 7 ± 1 4	0.67	89 4 ± 4.0	59.3 ± 4 6	0.66

Thymuses were pooled from 5 10 animals and the weight of the individual thymuses was calculated.

ly 35 per cent in the two groups of older mice and about 50 per cent in the group of newborn mice.

Histology Figs. 1–9 show the typical architecture of the thymus cortex of mice killed 6 and 24 h after injection of cortisol. Already 6 h after injection, the histology of the cortex was fundamentally altered, being of a moth-eaten appearance due to accumulation of pyknotic lymphocytes around cortico-epithelial cells the cytoplasm of which in many cases was loaded with nuclear debris. This change was most pronounced in the group of new-born animals and to a lesser extent in the group comprising 30 days old mice. In 6 days old mice pyknotic accumulations were only sparsely recognized Twenty-four hours after injection, the thymuses of new-born animals showed massive diffuse pyknosis all over the cortical and medullary areas, whereas pyknotic cells at this time were nearly absent in the group of 6 and 30 days old mice. However the thymus of mice in these two groups were more or less depleted of lymphoid cells, as indicated in Figs. 6 and 9

Differential counts. Table 2 shows the percentage of labelled thymic lymphoid cells incubated for 1 h with ^{3}H TdR. It is evident that only cells with a nuclear diameter $\geq 8\ \mu$ are labelled in all the age groups studied. This indicates that this cell group contains the population of thymic blast cells whereas cells with nuclear diameter $< 8\ \mu$ are non-proliferating small thymocytes.

Table 3 shows the percentage of small non-proliferating thymocytes, thymic blast cells (see above) and pyknotic cells 6 and 24 h after injection of cortisol. Cortisol-induced changes were evident already in the 6 h groups. Especially in the groups of new-born and 30 days old mice the per cent of pyknotic lymphocytes increased markedly (from < 1 per cent to about 20 per cent) In these two groups, the number of blast cells was reduced by 75–85 per cent of control values whereas the number of small lymphocytes was reduced only by about 15 per cent. In the light of the constant thymus weights—and the probably constant cell numbers—during the first 6 h, this finding strongly indicates that the blast cells have given rise to the majority of pyknotic cells 6 h after cortisol injection. In the 6 days group, the value of pyknotic cells had increased from 0.4 per cent up to 9.5 per cent, the increase obviously being due to cell destruction both of small thymocytes and thymic blast cells. Twenty four h after cortisol injection—when 35–55 per cent of the

TABLE 2 *Percentage of Labelled Thymocyt s Incubated for 1 Hou with H TdR. Each Val e R presents the Mean of 3 Animals*

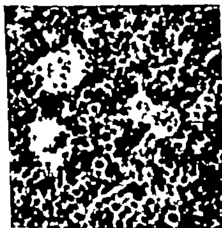
Animal age	Labelled lymphoid cells in per cent: nuclear diameter $< 8\ \mu$	nuclear diameter $\geq 8\ \mu$
12 h	0 2	56.4
6 days	0.5	66.3
30 days	0.5	59 7

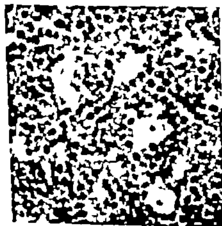
3 thymuses were pooled.



Figs 1–9 Typical cortical areas of thym from new-born mice (1 3) 6 days old mice (4–6) and 50 days old mice (7–9) Figs. 1 4 and 7 are controls. Figs. 2 5 and 8 are thymuses removed 6 h after injection of cortisol. Figs. 3 6 and 9 are thymuses removed 24 h after injection. Note the areas in which massive pyknosis occurred 6 h after injection (arrows) The horizontal bar represents 20 microns.







cells were lost from the thymus as judged from the decreased weight of the organ—only about 1 per cent pyknoses were found in thymus smears from 6 and 30 days old animals. At this time about 50 per cent of the lymphocytes in thymus of new-born mice were found to be pyknotic. Thus, the differential counts strongly support the histological findings. In all three age groups, the differential counts revealed that thymic blast cells are more sensitive to the cytotoxic action of cortisol than small thymocytes both 6 and 24 h after injection.

Supravital dye exclusion. Table 4 shows the results of nigrosin-dye exclusion performed on thymus single-cell suspensions. It is seen that both new-born and 30 days old animals showed increased percentages of non-viable (stained) cells 6 h after cortisol injection whereas the 6 days old mice were not influenced by treatment. Twenty-four hours after injection, the percentage of non-viable thymocytes obtained from 6 and 30 days old mice was at control levels. The extensive pyknosis in the group of new-born mice made it impossible to test this group 24 h after injection.

Incorporation of H TdR and H UdR. Fig. 10 demonstrates the effect of cortisol treatment *in vivo* on the incorporation of ^{3}H TdR and ^{3}H UdR of thymocytes obtained 6 and 24 h after injection. It is evident from Fig. 10 that incorporation of ^{3}H TdR 6 h after injection of cortisol was inhibited to the same degree (app. 40 per cent) in the three groups of animals. Twenty-four hours after injection, the inhibition of ^{3}H TdR incorporation was most pronounced in the thymus of new-born animals (app. 84 per cent) being app. 70 per cent and app. 50 per cent in the 6 and 30 days old mice, respectively. The incorporation of ^{3}H UdR was inhibited in all three groups already 6 h after injection of cortisol being most pronounced in the group of newborn animals (app. 60 per cent) and least pronounced in the 6 days old mice (app. 35 per cent). Twenty-four hours after injection, it applies to new-born as well as to, 6 days old mice that inhibition was about

TABLE 3 *Mean Percentage Cell Distribution at 6 and 24 h after Administration of Cortisol. Each Value Represents the Mean of Values Obtained in 3–5 Animals. The Values of S.E.M. are Recorded in the Table. Two Thousand Consecutive Cells from Each Thymic Smear Were Counted*

Animal age	Mean percentage lymphoid cell distribution								
	Nuclear diameter <8 μ			Nuclear diameter ≥8 μ			Pyknotic cells		
	Control	Time after injection		Control	Time after injection		Control	Time after injection	
		6 h	24 h		6 h	24 h		6 h	24 h
New-born *	87 6	75 1	48 1	12 6	3 8	3 7	0 8	21 1	48.2
6 days	80 4±0.5	74.5±0 8	89 7±3 1	19 2±0.5	16 0±1 0	9 1±3 0	0 4±0 1	9.5±0 1	1 2±0 1
30 days	89.9±2 6	77 8±4 7	93.2±0 7	10.3±2 4	1 7±0 6	5.8±0 7	0 6±0 1	20.5±4 7	1 0±0 3

* Injected with saline, values pooled from 6 and 24 h groups.
5 thymuses were pooled at each time interval.

TABLE 4. *Percentage of Non-viable Thymocytes Registered by Supravital Nigrosin-dye Exclusion. Each Value Represents the Mean of Values Obtained i 3–5 Animals. The Values of S.E.M. A e Recorded in the Table*

Animal age	Per cent non-viable thymocytes			
	Control		Cortisol treated	
	6 h	24 h	6 h	24 h
New-born	5.7	8.1	12.5	—**
6 days	4.3±0.7	5.7±0.7	5.8±0.6	6.3±0.6
30 days	3.2±0.4	4.9±0.2	6.5±0.5	5.8±1.1

5 thymuses were pooled at each time interval

** not counted because of the extensiv pyknosis.

50 per cent whereas 30 days old animals showed only 30 per cent inhibition of ^{3}H UdR incorporation.

In vitro effects of cortisol on the in orporation of H TdR and H UdR. The results of these studies are shown in Figs. 11 and 12 and in Table 5. Incubation of thymocytes with 10^{-4}M cortisol for from 2 to 6 h showed the same degree of inhibition of DNA-synthesis in the three age groups studied whereas incubation with 10^{-4}M cortisol resulted in the highest degree of inhibition in the two older age groups. In contrast, the inhibition of RNA-synthesis (measured by H UdR incorporation) was most pronounced in the group of new-born animals at the two concentrations of cortisol studied. At the highest concentration of cortisol (10^{-4}M) the inhibition of RNA-synthesis after 4 and 6 h incubation was far less pronounced in the 6 days

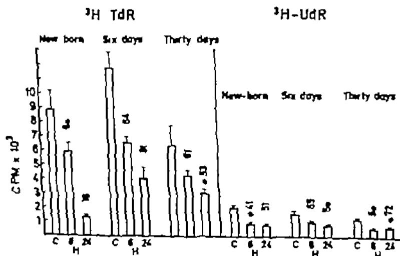


Fig. 10. Counts per minute (CPM) per 0.5 × 10⁶ thymocytes obtained from animals which had received one injection of saline (C) or cortisol 6 and 24 h prior to sacrifice. Thymocytes were incubated for 1 h with ^{3}H TdR or ^{3}H UdR, respectively. Values of S.E.M. are indicated. Each column represents the mean of values obtained in 3–5 animals except for thymuses of new-born animals where 5–10 thymuses were pooled at each time interval. Thymocytes from one animal were set up in triplicate cultures. The degrees of inhibition expressed as $\frac{\text{cortisol treated}}{\text{saline treated}}$ are recorded in the figure.

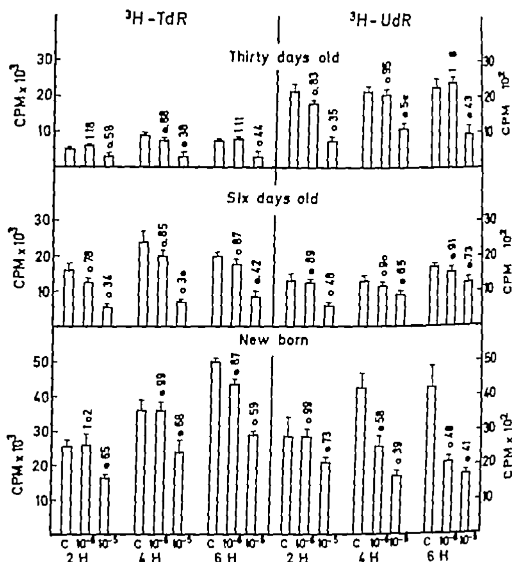


Fig 11 CPM per 0.5×10^4 thymocytes obtained from pools of 3–5 thymuses from mice of different ages. Each column represents the mean of 12 cultures. The cells were incubated for from 2 to 6 h with ^{3}H TdR or ^{3}H UdR added to the culture medium alone or to medium containing 10^{-5} or 10^{-6} M cortisol. Values of S.E.M. are indicated. The degrees of inhibition are recorded in the figure.

old animals than in new born and 30 days old animals.

Table 5 shows the *viable* cell numbers determined by nigrosin dye exclusion in cultures incubated for 6 h in 10^{-5} and 10^{-6} M cortisol. In all age groups studied 10^{-6} M cortisol tend to increase the viable cell number whereas 10^{-5} M cortisol obviously decreased the number of viable cells by about 10–25 per cent. In Fig 12 the incorporation of ^{3}H TdR and ^{3}H UdR after 6 h of incubation has been recorded as a function of *viable* cells using the data in Fig 11 and Table 5. The data in Fig 12 strongly indicate that the RNA synthesis of viable thymocytes from 6 days old mice are more resistant to the cortisol-induced inhibitory effect than the RNA-synthesis of thymocytes in the new born mice and in 30 days old animals.

DISCUSSION

The present results indicate that thymus lymphocytes of mice of different ages exhibit different degrees of sensitivity to the lymphocytotoxic action of cortisol *in vivo* as well as *in vitro*, the thymocytes of new-born mice being most sensitive and the thymocytes of 6

ABLE 5. Number of Viable Thymocytes in Culture Incubated fo 6 h with Different Concentrations Cortisol. Thymocytes Wer Obtained from Pools of Three Thymuses at Each Age Interval. Individual Data R present the Mean of Three Cultures

Concentration of cortisol	New-born × 10⁶	Six days old × 10⁶	Thirty days old × 10⁶
0	1.15±0.25	0.52±0.08	0.48±0.09
10 M	1.52±0.23	0.56±0.05	0.56±0.11
10 M	0.86±0.17	0.48±0.10	0.58±0.07

$\pm$ S.E.M.

ys old mice being least sensitive to cortisol. hymus weight decreases, occurrence of thym- pyknoses and non-viable cells in supravitally ained preparations, and inhibition of DNA nd RNA-synthesis measured by incorpora on of H TdR and H UdR respectively *in vivo* and *in vitro* are parameters which have een used in previous studies on cortisol-mediated lymphoid involution (3, 7 8, 10 12, 13 16) The high rate of cortisol-induced pyknoses and karyorrhexis of thymocytes obtained from new born mice compared to that of thymocytes of 6 days old mice probably reflects different stages of maturation of thymocytes during the process of ontogenesis. In fact, early age dependent patterns of cellular differentiation of mouse thymocytes with respect to thymocyte size distribution (18) and functional capacities (2, 4 17) have been reported. From the age of one month and onward these differences more or less disappear (4 18) Differential counts on thymic smears and histology of thymus sections revealed both massive intrathymic cell death of cortisol treated new-born mice compared with that of 6 and 30 days old animals. In spite of the fact that the thymus weight decreased by 35 per cent 24 h after cortisol administration, pyknoses and cell death were only sparsely recognized in 6 and 30 days old animals, the phenomena being very intensive in new born mice. Thus, cortisol-induced lymphocyte depletion in the older thymuses may reflect both cell destruction *in situ* and an increased rate of thymocyte emigration induced by cortisol treatment.

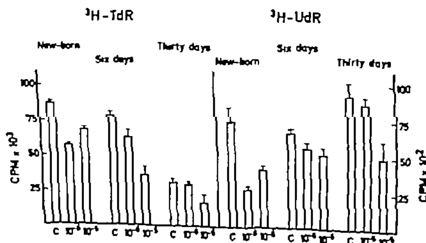


Fig. 12 CPM per 0.5 × 10⁶ viable thymocytes. The data are calculated from the 6 h-values in Fig. 11 and the results in Table 4. For details see text.

It has been shown in previous studies that cortisol induces inhibition of incorporation of ^{3}H TdR into nuclear DNA and of ^{3}H UdR into RNA of lymphoid cells from different species, the effect being most pronounced in mouse and rat thymocytes (3 12 16) This was confirmed in the present study in the three age groups examined However, the data indicate that the inhibition induced by cortisol is strongly dependent upon the age of the mouse. Thus, 24 hours after cortisol administration the DNA-synthesis of thymocytes in the new born was far more inhibited than DNA synthesis of thymocytes from 6 and 30 days old animals. The differential counts which at this stage showed intensive destruction of thymic blast cells in the new born are in agreement with this finding Since RNA synthesis occurs in almost 100 per cent of murine thymocytes as judged from autoradiographical studies (author's unpublished results) the cortisol induced inhibition of RNA-synthesis of thymocytes probably reflects a hormonal effect on thymic blast cells and on non proliferating small thymocytes. After treatment *in vivo* it was found that thymocytes in new-born and 6 days old animals were more susceptible to cortisol induced inhibitor effect of RNA-synthesis than thymocytes from 30 days old mice.

The inhibitory effect of cortisol on DNA and RNA synthesis of thymocytes *in vitro* was evident in the three age groups studied, using concentrations of cortisol close to pharmacological and physiological plasma levels, respectively (8) Since about 90 per cent of the circulating corticoid are bound to transcortin and albumin in the plasma (19) we used culture medium without addition of serum This is probably the reason why recovery of viable cells was poor during the 6 h period of incubation (cf Table 5 and (9)) The inhibitory effect of 10^{-6}M cortisol on the DNA synthesis was nearly identical in the three age groups studied In contrast to the *in vivo* findings, the effect of 10^{-6}M cortisol was more pronounced in the groups of 6 and 30 days old animals than in the group of new-born mice This was also true when DNA-depression was expressed as function of viable cells only The poor recovery of viable cells in cultures of thymocytes of "older" mice might explain this discrepancy If thymocytes were incubated for 4 and 6 h with 10^{-8}M cortisol, inhibition of RNA synthesis would be most pronounced in new born and 30 days old mice. This difference was also marked when RNA-synthesis was expressed as a function of viable cells only In contrast to this finding, any differences between the cytolytic action of cortisol *in vitro* upon 6 and 22 weeks "old" thymocytes, respectively were not observed (9)

As mentioned above, the recovery of viable cells in thymocyte cultures incubated for 6 h was very poor The data showed, however that neonatal thymocytes are more resistant to culture environments than thymocytes obtained from mice aged 6 and 30 days. This fact might explain some of the functional differences between new born and older thymocytes observed *in vitro*

The terms "cortisol sensitive" *versus* cortisol resistant" have been used currently in the literature to define subpopulation of thymocytes within the thymus (1 6, 10 20) "Cortisol sensitive" thymocytes constitute about 90 per cent of all thymic lymphocytes, they are localized predominantly in the cortex, and disappear within 48 h after treatment with 50–250 µg cortisol per g mouse or rat. The remaining 10 per cent are thymocytes which are localized predominantly in the medulla and they remain obviously unaffected by treatment —they are "cortisol-resistant The present data suggest that the disappearance of "cortisol-sensitive" thymocytes after cortisol treatment in vivo from the thymus of different age groups might be due not only to a different susceptibility to cortisol induced cell destruction *in situ* but also to an age dependent difference in cortisol-mediated thymocyte emigration This view is in accordance with that obtained in studies of guinea pigs which indicate that cortisol treatment increases the export of cells from the thymus (14)

REFERENCES

1 *Blomgren H & A dersson B* E idence for a small pool of immunocompetent cells in the mouse thymus. Exp. Cell. Res. 57 185–192 1969

2 *vonBoehmer H & Byrd W J* Responsiveness of thymus cells to syngeneic and allogeneic cells. Nature New Biol. 235 50–52, 1972.

3 *Brunk Johnsen T & Dougherty T F.* Incorporation of adenine-8-C[14] into RNA and DNA of mouse lymphatic tissue. Proc Am. Assoc Cancer Research 3 9 1959.

4 *Byrd, W J vonBoehmer H & Rouse B. T* The role of thymus in maturational development of phytohemagglutinin and pokeweed mitogen responsiveness. Cell. Immunol. 6 12–24 1973

5. *Claësson M H & Hartmann, N R* Cytodynamics in the thymus of young adult mice. A quantitative study on the loss of thymic blast cells and nonproliferating small thymocytes. Cell Tissue Kinet 1976, in press.

6. *Claësson M H., Hardt F & Röpke C* Graft-versus-host reactions mediated by thymus-bone marrow combination, spleen cells, and lymph node cells from amyloidotic and nonamyloidotic mice. Acta path. microbiol. scand. Sect. B 82 719–723 1974

7 *Claësson M H & Röpke C* Quantitative studies on cortisol-induced decay of lymphoid cells in the thymolymphatic system. Acta path. microbiol. scand. 76 376–382, 1969

8 Claman H N Corticosteroids and lymphoid cells New Eng J Med. 287 388–397 1972.

9 *Claman H N Moorhead J W & Benner W H* Corticosteroids and lymphoid cells in itro I Hydrocortisone lysis of human, guinea pig, and mouse thymus cell J Lab Clin. Med 78 499–507 1971

10. *Cohen J J Fischbach, M & Claman H N* Hydrocortisone resistance of graft-versus-host activity in mouse thymus, spleen, and bone marrow J Immunol. 105 1146–1150, 1970.

11 *Dougherty T F Berliner M L Schneebeli G L & Berliner D L* Hormonal control of lymphatic structure and function. Ann. N Y Acad. Sci. 113: 825–843 1964

12. *Drews J* The effect of prednisolone injected in vi o on RNA synthesis in rat thymus cells. Europ. J Biochem. 7 200–208, 1969

13. *Ishidate M J & Metcalf D.* The pattern of lymphopoiesis in the mouse thymus after cortisone administration or adrenalectomy Austral J Exp. Biol. Med. Sci. 41 637–649 1963

14 *Larsson, B.:* Export and import of H-thymidine-labelled lymphocytes in the thymus of normal and steroid-treated guinea-pigs. Acta path. microbiol. scand. 70 390–397 1967

15 *Landis P M & Schek U.* The effects of steroids on the histology and ultrastructure of lymphoid tissue. III. Thymus in prolonged steroid induced involution. Pathol. Eur. 4 58–68, 1969

16. *Makman M H., Dvorkin B & White A.* Influence of cortisol on the utilization of precursors of nucleic acids and protein by lymphoid cells in vitro J Biol. Chem. 7 1485–1497 1968.

17 *Mosier D E.* Ontogeny of mouse lymphocyte function I Paradoxical elevation of reactivity to allogenic cells and phytohemagglutinin in Balb/c fetal thymocytes. J Immunol. 112 305–310, 1974

18. *Salinas, F A., Smith L H & Goodman, J W* Cell size distribution in thymus as a function of age. J Cell. Physiol. 80 339–346, 1972.

19 *Sawin C T.* The Hormones. Endocrine Physiology Little, Brown and Comp Boston 1969

20 Tigelaar R. E. & *Asofsky R.* Graft-versus-host reactivity of mouse thymocytes Effect of cortisone pretreatment of donors. J Immunol. 110 567–574 1973

21 *Whitfield J F Perris, A D & Youdale T.* Destruction of nuclear morphology of thymic lymphocytes by the corticosteroid cortisol. Exp Cell Res. 52 349–362, 1968.

Acta path. microbiol scand Sect. C, 84 196–202 1976

PURIFICATION OF IMMUNOGLOBULIN G Fc-REACTIVE FACTOR FROM *STREPTOCOCCUS AZGAZARDAH*

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Christensen, P & Holm, S E. Purification of Immunoglobulin G Fc reactive factor from *Streptococcus azgazardah* Acta path. microbiol scand Sect. C, 84 196–202 1976.

A factor from *Streptococcus azgazardah* (Lancefield's group C) capable of reacting with the Fc fragment of human IgG was partially purified and characterized. The bacteria were lysed by infection with phage and the IgG Fc reactive factor was purified from the crude *lysate* by affinity chromatography on a IgG-coupled Sepharose 4B column. The column was eluted with 3 M KSCN and the eluate gel filtered on a Sephadex G 75 column. A 30-fold purification was obtained The molecular weight of the IgG Fc reactive factor was estimated to be 60 000 The factor was sensitive to trypsin treatment and to heating 95 C, 10 min at pH 2.0 On polyacrylamide electrophoresis only *one* band was obtained. The trypsin- and heat sensitivity and the estimated molecular weight of the factor was similar to an IgG Fc reactive factor preparation obtained from group A, type M 56 streptococci by heat treatment at 120 C for 30 min.

Key words Streptococci IgG Fc fragment purification

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The existence of a non immune interaction between streptococci and immunoglobulin i e interaction without involving the antibody combining sites, can be inferred from the capacity of streptococci to agglutinate sheep red cells sensitized with a subagglutinating dose of rabbit anti sheep red cell antibodies (*Kronvall* 1973 *Christensen & Kronvall* (1974) the uptake of [125]I labelled human myeloma proteins (*Christensen & Oxelius* 1974 and 1975), agglutination of streptococci using soluble immune complexes (*Christensen* 1975) and in the electrone microscope by the use of human IgG myeloma protein visualized with ferritin-conjugated rabbit anti human IgG antibodies (*Christensen et al* 1976a) Evidence for a reactivity with the Fc region of IgG by a surface component on streptococci has recently been obtained (*Christensen et al* 1976b)

The present investigations concern the purification and characterization of the Fc reactive factor from *Streptococcus azgazardah* We utilized the capacity of phage C to lyse *S azgazardah* by inducing production of the phage-associated lysin. This enzyme lyses the walls of group A C and E streptococci (*Evans 1934 Maxted 1957 Krause 1957*) The Fc reactive factor was also released from

streptococci group A, type M 56 by treating the cells at 120 C for 30 min in order to obtain material for comparison with the component obtained by phage lysis. The IgG Fc reactive factor from *S agazardak* was isolated by affinity chromatography on a column of human IgG-coupled Sepharose 4 B purified further by gel filtration and characterized.

MATERIALS AND METHODS

Streptococcal Strains

The following strains were used *Streptococcus pyogenes* group A, type M 1 (Colindale No. 8198) type M 12 (Col. No. 100083) and type M 56 (Col. No. 100191) and *S agazardak* group C (*Evans* 1934) The streptococci were grown on trypticase-yeast autolysate medium (*Holm & Falsén* 1967)

Demonstration of Streptococcal IgG Fc-Reactive Factor

The solubilized Fc-reactive factor from streptococci was quantified by testing the capacity of such preparations to inhibit the uptake of radio-labelled IgG myeloma protein by intact streptococci (*Christensen & Oxelius* 1974) A suspension of about 5×10^{8} streptococci in 0 2 ml of phosphate buffered saline (PBS, 0.12 M NaCl, 0 03 M phosphate, pH 7 2) (*Christensen & Oxelius* 1974) was added to mixture of the test sample and 1 µg ^{125}I labelled IgG myeloma protein. The uptake of labelled IgG myeloma protein (expressed as percentage of radioactivity added) was compared with the maximal uptake measured in control experiments with the same amount of streptococci, but without addition of IgG Fc-reactive preparations. One unit of IgG Fc-reactive factor was defined as the minimum amount inhibiting the uptake of ^{125}I labelled IgG myeloma protein group A, type M 1 streptococci to 50 per cent of the maximal uptake A standard curve for the inhibitory capacity was obtained by addition of different dilutions of purified Fc-reactive factor from *S agazardak* (Fig 1)

Immunosorbent and Gel Filtration Columns

Commercial, pooled human IgG was coupled to Sepharose 4 B using CNBr (*Axén et al* 1967 *Cuatrecasas & Anfinsen* 1967) Chromatography was performed on Sephadex G-75 columns (*Flodin & Killander* 1962) Sepharose 4 B and Sephadex G-75 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. The molecular weight of the IgG Fc-reactive factor was estimated by Sephadex G-75 gel filtration experiments as described

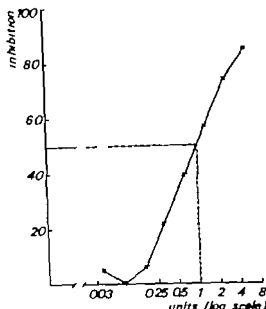


Fig 1 Standard curve for determination of the content of IgG Fc-reactive factor in streptococcal preparations One unit IgG Fc-reactive factor was defined as the minimum amount capable of inhibiting the uptake of ^{125}I labelled IgG myeloma protein (1 µg added) on 0.2 ml standard suspension of group A, type M 1 streptococci to 50 per cent of the maximal uptake obtained without addition of IgG Fc-reactive factor The standard curve was obtained with different dilutions of purified *S agazardak* IgG Fc-reactive factor The content of IgG Fc-reactive factor in the sample to be tested was calculated from the dilutions giving inhibition corresponding to 0.25–2 units. Ordinate: differences between the uptake of ^{125}I labelled IgG myeloma protein with and without IgG Fc-reactive factor as percentage of the uptake obtained without addition of IgG Fc-reactive factor

by *Andrews* (1964) Calibrating markers, cytochrome C, chymotrypsinogen A, egg albumin, bovine serum albumin, aldolase and catalase were purchased from Boehringer Mannheim, GmbH (W Germany)

Trypsin Treatment of IgG Fc Reactive Factor from Streptococci

Trypsin treatment of IgG Fc-reactive factor preparations was performed by mixing equal volumes of such preparations (dissolved in PBS) and trypsin (Sigma, St. Louis, MO., U.S.A., 1 mg/ml tris (hydroxymethyl) aminomethane (Tris) 0.2 M, pH 8.0) followed by incubation at 37° C for 1 h.

Trypsin treatment was stopped using soybean trypsin inhibitor (Sigma) Trypsin trypsin inhibitor or mixtures of these preparations were not capable to inhibit the uptake of ^{125}I labelled IgG myeloma protein by streptococci

Protein Determination

Protein concentrations were measured with a modification of Folin's method (*Lowry et al.* 1951)

Polyacrylamide Electrophoresis

Disc electrophoresis in polyacrylamide gel was performed as described by *Davis* (1964)

Sensitized Sheep Red Cells and Human Blood Red Cells Coated with Anti D Antibodies

Sheep red cells were coated with half the minimal agglutinating dose of rabbit anti-sheep red cell antibodies. Human Rh positive blood red cells were coated with anti-D antibodies from a woman immunized by pregnancy as described by *Grubb & Laurell* (1956) (the anti D antibodies belonging to Gm (1))

Immunoglobulin Preparations

IgG myeloma protein was purified as described earlier (*Christensen & Oxelius 1974*) The myeloma protein was labelled with ^{125}I as described by *McConahey & Dixon* (1966) Commercial pooled human IgG was purchased from AB Kabi, Sweden (batch No. 44791)

Solubilization of IgG Fc-Reactive Factor by Treatment of S argazardah with Phage C

S argazardah was infected with phage C as described by *Fischetti et al.* (1971) 1 l of *S argazardah* culture was allowed to grow to an optical density of 0 25 at 540 nm (1 cm cuvette) Phage C preparation, 10 ml, containing about 10^{9} plaque forming units/ml was added After 30 min at 37 C the bacterial suspension was chilled in ice and the streptococci centrifuged at 3000 *g* The bacterial pellet was suspended in 15 ml 0 05 M phosphate buffer pH 6 1 and incubated again at 37 C until it had undergone lysis. *S argazardah* and phage C were kindly supplied by Dr E. *Kjems* the State Serum Institute Copenhagen

Solubilization of IgG Fc Reactive Factor by Heat Treatment of Streptococcus Group A Type M 56

The sediment from a culture of group A M 56 streptococci was suspended in PBS and autoclaved at 120 C for 30 min. The bacterial suspension was then centrifuged at 3000 *g* and the supernatant sterile filtered.

RESULTS

Solubilization of IgG Fc Reactive Factor from Streptococci

Heating of the sediment from 20 l streptococcus group A, type M 56 culture suspended in PBS at 120° C for 30 min in an autoclave gave a solution with a total protein content of 725 mg containing 256 units of IgG Fc reactive factor The solution (concentrated to 10 ml) agglutinated sensitized sheep red cells in a dilution of 1 3700 Human blood red cells coated with anti-D antibodies were agglutinated in a dilution of 1 1000 No agglutinating capacity or inhibition of the uptake of ^{125}I labelled IgG myeloma protein on group A, type M 1 streptococci was found in the solution after autoclaving group A type M 1 and M 12 streptococci or *S argazardah* Other solubilization procedures were tried for group A type M 1 M 12 and M 56 and *S argazardah* i e. extraction with HCl at pH 2.0 NaOH at pH 10 0 PBS at pH 7.2, all at 100° C, 10 min or 1 hour Very low activity was, however obtained in the agglutination test and the inhibition test with ^{125}I labelled IgG myeloma protein and group A, type M 1 streptococci After formamide extraction no IgG Fc-reactive factor was demonstrable in the streptococcal residue washed with PBS or in the formamide extract dialyzed against PBS (formamide extraction performed as described by *Fuller* 1938) Ultrasonic treatment and X pressing as well as treatment in the Mickle disintegrator yielded only small quantities of the factor

Lysis of a 250 ml culture *S argazardah* with an OD of 0.25 with phage gave a preparation containing 128.8 mg protein and 640 units of streptococcal IgG Fc reactive factor (Table 1) When the IgG Fc reactive factor was removed from the lysate of *S argazardah* by affinity chromatography lysis of M 1 and M 56 streptococci was possible by treatment of these streptococci with solution, still containing phage associated lysin. Concentrations of IgG Fc reactive factor similar to those obtained by lysis of *S argazardah* were found by lysis of M 1 and M 56

TABLE 1 *Purification of the Streptococcal IgG Fc Reactive Factor from S agacardah*

	Units IgG Fc-reactive factor*	Protein content (mg)	Units IgG Fc-reactive factor/mg protein
Preparation of *S agacardah* obtained by lysis with phage C	640	128.8	5.0
Eluate from IgG coupled Sepharose 4 B column obtained with PBS (Fig. 2 pool I)	4	80.6	0.05
Eluate from IgG coupled Sepharose 4 B column obtained with 3 M KSCN (Fig 2 pool II)	163	2.6	62.7
Further purification by gel filtration on Sephadex G 75 (Fig. 3 pool III)	114	0.78	146.1

* For definition, see text to Fig. 1

streptococci. None of the IgG Fc-reactive preparations from strains M 1 and M 56 obtained by phage associated lysin were capable of agglutinating sensitized sheep red cells or human blood red cells coated with anti-D antibodies.

Purification of Streptococcal IgG Fc reactive Factor from S. agacardah

Preparations of IgG Fc reactive factor obtained by lysis of *S agacardah* were applied to a column of human IgG-coupled Sepharose 4 B. After washing with PBS adsorbed material was eluted with 3 M KSCN (*Edgington* 1971) (Fig. 2) The eluate was dialyzed against PBS and the solution was concentrated in an ultrafiltration cell (Diaflo UM 10) The inhibiting capacity resided in the eluate obtained with 3 M KSCN (Table 1)

Further purification was obtained by gel filtration on Sephadex G 75 column. The inhibitory was found in the first half of the first peak (Fig 3) Polyacrylamide electrophoresis showed only one band (63 µg protein applied)

After gel filtration the content of IgG Fc reactive factor was 146 1 units/mg protein (Table 1) We had thus obtained a 30-fold purification of the IgG Fc-reactive factor from the protein material in the crude lysate of *S agacardah*

Characterization of the Streptococcal IgG Fc Reactive Factor

The molecular weight of the streptococcal IgG Fc-reactive factor obtained by lysis of *S agacardah* or autoclaving of group A, type

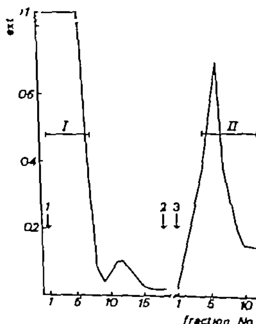


Fig 2 Elution of the streptococcal IgG Fc-reactive factor from IgG coupled Sepharose 4 B column 1 eluate obtained with PBS; 2. indicates thorough washing with PBS; 3 fractions obtained by elution with 3 M KSCN I and II indicate fractions pooled and concentrated for further studies.

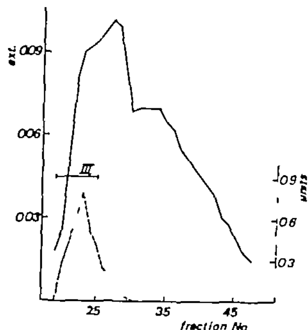


Fig 3 Gel filtration on Sephadex G 75 column of pool *II* (*Fig. 2*) Dotted line IgG Fc reactive factor units per ml Unbroken line absorbancy at 280 nm of the fractions.

M 56 streptococci was estimated to be about 60 000 by chromatography on calibrated Sephadex G 75 column The inhibiting capacity of the *S azgazardah* IgG Fc reactive factor preparation and the capacity to agglutinate sensitized sheep red cells of the autoclaved extract of M 56 were completely destroyed by trypsin treatment and by heating at 95 C, 10 min at pH 2 0 followed by neutralization with NaOH

The streptococcal IgG Fc reactive factor from *S azgazardah* could not inhibit the uptake of [125]I labelled IgG myeloma protein on *S azgazardah* on the contrary on addition of factor preparation the uptake on *S azgazardah* increased from 44 0 per cent (of 1 µg [125]I labelled IgG myeloma protein added) to 68.9 per cent on 0 2 ml *S azgazardah* standard suspension If the factor preparation was added to the streptococci followed by washing in PBS a similar increase in uptake of [125]I labelled IgG myeloma protein was found. On the other hand inhibitory effect of the *S azgazardah* factor was obtained by assessing the uptake on standard suspensions of M 1 M 12 or M 56 streptococci of [125]I labelled IgG

DISCUSSION

The group C phage associated lysin has been used for various purposes in the study of the streptococcal cell layers. The enzyme is now known to be an N-acetyl muramyl-L-alanine amidase attacking the alanine muramic acid linkage (*Zabriskie et al.* 1972) Treatment of isolated cell walls gave a higher molecular weight material containing mainly the group-specific carbohydrate with a portion of mucopeptide still attached To some extent other materials of lower molecular weight containing the mucopeptide moiety free from carbohydrate but rich in NH_2-terminal alanine was also obtained It has been suggested that such proteins as M R and T proteins, are attached to a polymer the G-polysaccharide, serving as an anchor point for the protein (*Barkulis et al* 1968) The lysin releases the proteins still coupled to this polysaccharide (*Ghuysen* 1968)

Unlike protein A from *Staphylococcus aureus* (*Kronvall* 1972, *Hjelm et al.* 1972) the IgG Fc reactive factor could not be obtained in pure form by affinity chromatography on an IgG-coupled Sepharose 4 B column The IgG coupled to the column was commercial, pooled human IgG which presumably contains antibodies to various streptococcal antigens (*see Halbert* 1964) Hence, streptococcal antigens could be retained on the column also by means of the antibody combining sites of IgG It was demonstrated by gel filtration on Sephadex G 75 column that the IgG Fc reactive factor constituted only a part of the material eluted with 3 M KSCN from the Sepharose 4 B coupled IgG column

The average molecular weight of M protein as estimated from sedimentation and viscosity data is about 40 000 (*Pappenheimer et al* 1942 *Fox* 1968) M proteins are thought to have a highly elongated structure since they emerge from the Sephadex G 200 columns almost at the void volume, indicating a molecular weight range of 100,000 or more (*Fox* 1968) The fact that the IgG Fc reactive factor is retained by UM 10 filtra

tion filters (Diaflo) but not XM 50 (*Christensen & Holm* unpublished observation) is in accordance with the estimated molecular weight found for the IgG Fc reactive factor 60,000.

The streptococcal IgG Fc reactive factor was highly sensitive to exposure to 95 C for 10 min, pH 2.0 a procedure used for extraction of M-protein from streptococci (*Lancefield & Perlman* 1952) Like M-protein, and unlike T-protein, it was sensitive to treatment with trypsin. We found earlier that extraction of streptococci at 100 C, 10 min at pH 2.0 released only small amounts of the factor while trypsin treatment of the intact streptococci diminished, but did not abolish, their capacity to interact with the Fc part of IgG (*Christensen & Kronvall* 1974 *Christensen & Oxelius* 1974) The possibility of the M-protein (MW 40,000) being part of the IgG Fc reactive factor molecule (MW 60,000) is refuted by the fact that M-proteins extracted with hot HCl and phage associated lysin equilibrate at approximately equal densities when centrifuged in sucrose gradients (*Fox* 1968) Furthermore, the streptococcal IgG Fc reactive factor from *S zygazardah* does not show the electrophoretic heterogeneity characteristic of the M-protein (*Fox* 1968) These findings do, however not exclude the possibility of the IgG Fc reactivity being connected with M or T protein on the streptococci.

We found that the IgG Fc reactive factor from *S a-gazardah* increased the uptake of [125]I labelled myeloma protein on *S zygazardah* but inhibited the uptake on some other streptococci. This was due to an affinity of the IgG Fc-reactive factor for the cell surface of the parent strain Similarly staphylococcal protein A from *S aureus* Cowan I labelled with [125]I has a clear affinity for the protein A negative strain, Wood 46 (*Christensen* unpublished observation) The results obtained with the *S a-gazardah* IgG Fc-reactive factor indicate differences between the IgG Fc-reactive factors among the various strains this possibility is receiving further attention. The difference between the *S zygazardah* IgG Fc-reactive factor and the group A, type M 56 factor in their ability to agglutinate sensitized sheep red cells could not be ascribed merely to differences in solubilization procedures, since only the M 56 factor resisted heat treatment at 120 C for 30 min. This finding might also indicate differences of the IgG Fc reactive factor from strain to strain.

REFERENCES

Andrews P Estimation of molecular weights of proteins by Sephadex gel-filtration. Biochem. J 91 222 233 1964

Axén R., Porath J & Ernback, S. Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. Nature 214 1302–1304 1967

Barkulis, S S Lok V Bolitchk J J & Smith C E. Attachment of type specific proteins to the wall of group A streptococci. Bacteriol. Proc. 1968, p. 110.

Christensen P Agglutinability of some selected streptococci by immune complexes. Acta path. microbiol. scand. Sect. C, 83 28–34 1975

Christensen P & Kronvall, G Capacity of group A, B, C, D and G streptococci to agglutinate sensitized sheep red cells. Acta path. microbiol. scand. Sect. B 82 19–24 1974

Christensen P & Oxelius, V A Quantitation of the uptake of human IgG by some streptococci groups A, B, C and G Acta path. microbiol. scand. Sect. B, 82 475–483, 1974

Christensen P & Oxelius V A A reaction between some streptococci and IgA myeloma proteins. Acta path. microbiol. scand. Sect. C, 83 184 188, 1975

Christensen P Sellers L. I & Oxelius V A Visualization of the binding of IgG myeloma proteins to some group A streptococci. Acta path. microbiol. scand. Sect. C, 84 203–209 1976 a.

Christensen P., Johansson B. G & Kronvall, G. Interaction of streptococci with the Fc fragment of IgG. Acta path. microbiol. scand. Sect. C, 84 73–76, 1976 b.

Cuatrecasas P & Anfinsen C B. Affinity chromatography Ann. Rev Biochem. 40 259–278, 1967

Davis B. J Disc electrophoresis. II Method and application to human serum proteins. Ann. N Y Acad. Sci. 121 404–427 1964

Edgington T S Dissociation of antibody from erythrocyte surfaces by chaotropic ions. J Immunol. 106 673–680 1971

Evans A C. Streptococcus bakterio-phage

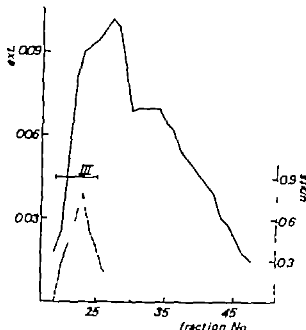


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The streptococcal IgG Fc reactive factor from *S azgazardah* could not inhibit the uptake of ^{125}I labelled IgG myeloma protein on *S azgazardah* on the contrary on addition of factor preparation the uptake on *S azgazardah* increased from 44 0 per cent (of 1 μg ^{125}I labelled IgC myeloma protein added) to 68.9 per cent on 0 2 ml *S azgazardah* standard suspension If the factor preparation was added to the streptococci followed by washing in PBS a similar increase in uptake of ^{125}I labelled IgG myeloma protein was found. On the other hand, inhibitory effect of the *S azgazardah* factor was obtained by assessing the uptake on standard suspensions of M 1 M 12 or M 56 streptococci of ^{125}I labelled IgG

DISCUSSION

The group C phage associated lysin has been used for various purposes in the study of the streptococcal cell layers. The enzyme is now known to be an N acetyl muramyl-L-alanine amidase attacking the alanine-muramic acid linkage (*Zabriskie et al* 1972) Treatment of isolated cell walls gave a higher molecular weight material containing mainly the group-specific carbohydrate with a portion of mucopeptide still attached To some extent other materials of lower molecular weight containing the mucopeptide moiety free from carbohydrate but rich in NH_2-terminal alanine was also obtained. It has been suggested that such proteins, as M R and T proteins, are attached to a polymer the G-polysaccharide, serving as an anchor point for the proteins (*Barkulis et al* 1968) The lysin releases the proteins still coupled to this polysaccharide (*Ghuysen* 1968)

Unlike protein A from *Staphylococcus aureus* (*Kronvall* 1972, *Hjelm et al* 1972) the IgG Fc reactive factor could not be obtained in pure form by affinity chromatography on an IgG-coupled Sepharose 4 B column. The IgG coupled to the column was commercial, pooled human IgG which presumably contains antibodies to various streptococcal antigens (*see Halbert* 1964) Hence, streptococcal antigens could be retained on the column also by means of the antibody combining sites of IgG It was demonstrated by gel filtration on Sephadex G 75 column that the IgG Fc reactive factor constituted only a part of the material eluted with 3 M KSCN from the Sepharose 4 B coupled IgG column

The average molecular weight of M protein as estimated from sedimentation and viscosity data is about 40 000 (*Pappenheimer et al* 1942 *Fox* 1968) M proteins are thought to have a highly elongated structure since they emerge from the Sephadex G 200 columns almost at the void volume, indicating a molecular weight range of 100,000 or more (*Fox* 1968) The fact that the IgG Fc reactive factor is retained by UM 10 filtra

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VISUALIZATION OF THE BINDING OF IgG MYELOMA PROTEINS TO SOME GROUP A STREPTOCOCCI

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Christensen, P., Soltesz, L. V. & Oxelius, V.-A. Visualization of the binding of IgG myeloma proteins to some group A streptococci. Acta path. microbiol. scand. Sect. C, 84: 203–209, 1976.

The interaction between streptococci and human IgG which does not involve the antibody combining sites, was visualized in the electron microscope by the use of human IgG myeloma proteins and ferritin labelled rabbit anti-human IgG antibodies. The streptococci were allowed to react with human IgG myeloma proteins and the human IgG was then marked by addition of ferritin labelled rabbit anti-human IgG antibodies. Sections of streptococci studied in electron microscopy showed localization of the ferritin on the surface of the cell wall of all the streptococci of type M 1, M 3, M 12 and M 56 studied. Similar results were obtained if the ferritin labelled rabbit anti-human IgG antibodies alone were allowed to react with the streptococci. The uptake of ferritin labelled rabbit anti-human IgG antibodies could be inhibited by addition of normal rabbit serum.

Key words: Streptococci; IgG; electron microscopy; cross reactions.

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An interaction between some streptococci and immunoglobulins, which does not involve the antibody combining sites, was described 1973 by *Kronvall*. The interaction occurs with IgG and also between some streptococci and IgA; no major differences have been found with respect to reactivity between the IgG subclasses 1, 2, 3 and 4 (*Christensen & Oxelius* 1974 and 1975). The IgG region participating in the reaction is the Fc part (*Christensen et al.* unpublished results). The capacity to interact is greater among beta hemolytic streptococci of groups A, C and G than among B and D streptococci (*Christensen et al.* 1975). So far no identity between the reactive sites for IgG Fc on the streptococci and any known streptococcal antigen or other streptococcal activities has been demonstrated.

The interaction between streptococci and immunoglobulins can be shown by different methods: 1) the capacity of some streptococci to agglutinate sheep red cells sensitized with a subagglutinating dose of anti-sheep red cell antibodies (*Christensen & Kronvall* 1974), 2) the uptake of [125]I labelled purified human myeloma proteins by some streptococci (*Christensen & Oxelius* 1974 and 1975) and 3) the capacity of some streptococci to be agglutinated by immune complexes (*Christensen* 1975). This paper deals with the electron microscopic visualization of the interaction by the use of human IgG myeloma proteins and

study of four serological types Pupl. Hlth Rep. (Wash.) *49* 1386–1392 1934

Fichetti V A Gotschlich E. C & Berhnheimer A B Purification and physical properties of group C streptococcal phage-associated lysin. J Exp Med *133* 1105–1117 1971

Flodin P & Killander J Fractionation of human serum proteins by gel filtration Biochem. biophys Acta (Amst.) *63* 403–410 1962

Fox E N Purification and properties of group A streptococcal M protein. In Caravano R. (Ed.) Current research on group A streptococcus. Excerpta Medica Foundation. Amsterdam New York, London Paris, Milan, Tokyo Buenos Aires, 1968 p 68–74

Fuller A T The formamide method for the extraction of polysaccharides from haemolytic streptococci, Brit. J Exper Pathol. *19* 130–139 1938

Ghuysen J M Use of bacteriological enzymes in determination of wall structure and their role in cell metabolism. Bact Reviews *32* 425–464 1968.

Grubb R & Laurell A B Heriditary serological human serum groups. Acta path. microbiol. scand *39* 390–398, 1956

Halbert S P Analysis of human streptococcal infections by immunodiffusion studies of the antibody response. In Uhr J W (Ed.) The streptococcus rheumatic fever and glomerulonephritis. The Williams & Wilkins Company Baltimore 1964 p. 83–139

Hjelm H Hjelm K & Sjöquist J Protein A from *Staphylococcus aureus*. Its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobulins. FEBS Letters *28* 73–76 197?

Holm S E & Falsén E. An antigen-free medium for cultivation of beta-hemolytic streptococci. Acta path. microbiol. scand. *69* 264 276, 1967

Krause R M Studies on bacteriophages of hemolytic streptococci. I Factor influencing the interaction of phage and susceptible host cell. J Exp Med. *106* 365–384 1957

Kronvall G Purification of staphylococcal protein A using immunosorbents. Scand. J Immunol *1* 31–36 1972

Kronvall G A surface component in group A, C and G streptococci with non-immune reactivity for immunoglobulin G J Immunol. *111* 1401–1406 1973

Lancefield R C & Perlman G E. Preparation and properties of typespecific M antigen isolated from a group A type 1 hemolytic streptococcus. J Exp. Med. *96* 71–82, 1952.

Lowry O H Rosebrough N J., Farr L A. & Randall, R J Protein measurement with the Folin phenol reagent. J Biol. Chem. *193* 265–275 1951

Maxted W R The active agent in nascent phage lysis of streptococci. J Gen. Microbiol. *16.* 584–591 1957

McConahey P J & Dixon F J A method of trace iodination of proteins for immunologic studies Int. Arch. Allergy 29 185–189, 1966.

Pappenheimer A M Jr Williams J W & Zittle C A The antigenic structure of hemolytic streptococci of Lancefield group A. J Immunol. *43* 61–63 1942.

Zabriskie J B Read S E. & Fischetti V A Lysogeny in streptococci. In Wannamaker L W & Matsen, J M. Streptococci and streptococcal diseases. Academic Press, New York and London 1972, p. 101–116.

diamond knife (du Pont) mounted on a LKB-Ultratome III microtome.

All sections were examined with a Philips EM 300 electron microscope at 60 kV with the use of a 20 or 30 μm objective aperture. The micrographs are recorded on Kodak Fine Grain Release Positive (FGRP) film at a primary magnification of 9,000 × (Figs. 2, 3, 5, 6) or 18,000 × (Figs. 1 and 4). The film was developed with Kodak D 19-B developer.

RESULTS

The Uptake of ¹²⁵I Labelled IgG Myeloma Protein and Rabbit IgG by Some Group A Streptococci

The M+ve and M−ve variants of the same streptococcal types showed essentially the same uptake of ¹²⁵I labelled IgG myeloma protein, with the exception for the difference between the M+ve and M−ve variants of type M 1 (70 and 10 per cent, respectively of 1 μg IgG added to 0.2 ml standard suspension) (Table 1). The uptake of ¹²⁵I labelled rabbit IgG by M 1 (M+ve) streptococci was 65 per cent of the 1 μg added. By addition of 250 μg unlabelled human IgG myeloma protein, the uptake of rabbit IgG was 2 per cent; similarly unlabelled rabbit IgG 250 μg blocked the uptake of ¹²⁵I labelled IgG myeloma protein (the uptake was not measurable).

Electron Microscopic Studies

The ferritin was localized on the surface of the cell wall of type M 1 (M+ve) streptococci after addition of human IgG myeloma protein and ferritin labelled anti-human IgG antibodies (Fig. 1). Fig. 2 shows M 1 (M+ve) streptococci without addition of myeloma proteins or ferritin labelled rabbit anti-human IgG antibodies. Similar results were obtained with the other streptococcal strains. Figs. 3, 4 and 5 show type M 3 streptococci, (M+ve) (M−ve, R+ve) and (M+ve, R+ve) respectively: the three variants all showed presence of ferritin conjugated anti-human IgG antibodies on the surface after addition of IgG myeloma protein and the ferritin conjugated anti-IgG antibodies, regardless of the presence of M or R protein. The septa (or cross wall formations) were not ferritin covered (see for example Fig. 5). The results were similar with four different IgG myeloma proteins.

The uptake of rabbit anti-human IgG antibodies (ferritin conjugated) was negligable after previous addition of rabbit serum instead of human IgG myeloma protein. The uptake of ferritin labelled rabbit anti-human IgG antibodies on the surface of M 1 (M+ve) streptococci is shown in Fig. 6 (no human IgG myeloma protein added): the localization of ferritin is similar to that shown in Fig. 1.

The ferritin was also localized on the surface of type M 1 (M+ve) streptococci treated with IgG myeloma protein and anti-human IgG (ferritin conjugated) after previous treatment of the streptococci with *trypsin* (trypsin treatment diminishes the uptake of ¹²⁵I labelled IgG myeloma protein (*Christensen & Oxelius* 1974)).

TABLE 1. *The Uptake of ¹²⁵I Labelled IgG Myeloma Protein by Some Group A Streptococci*

	Streptococcal strains							
	M 1		M 3			M 12		M 36
	M+ve	M−ve	M+	M−ve R+	M+ve, R+ve	M+	M−ve	M+ve
The uptake of ¹²⁵I labelled IgG myeloma protein (per cent of 1 μg IgG added) by 0.2 ml streptococcal standard suspension	70	10	13	16	12	11	14	20

ferritin labelled rabbit anti human IgG anti bodies.

MATERIALS AND METHODS

Streptococcal Strains

The following strains were kindly supplied by dr *Maxted* the Central Public Health Laboratories, Colindale London group A type M 1 M protein positive (M+ve) (No 8198) M 3 (M+ve) (100064) M 3 (M−ve R protein positive (R+ve)) (100063) M 3 (M+ve R+ve) (D 58 X/11) M 12 (M+ve) and M 12 (M−ve) (1130) and M 56 (100101) Group A, strain S F 130 type T 1 (M−ve) was kindly supplied by dr *Perch* Statens Seruminstitut Copenhagen A standard suspension of about 2.5 × 10^{10} living streptococci per ml phosphate buffered saline (PBS 0 12 M NaCl, 0 03 M phosphate pH 7 2) was prepared for each strain as described previously (*Christensen & Oxelius* 1974)

Sera and Immunoglobulin Preparations

IgG myeloma proteins were isolated from the sera of four patients with multiple myeloma as described earlier (*Christensen & Oxelius* 1974) The isolated proteins (2.5 mg/ml) included only one subclass tested by immunoelectrophoresis (*Scheidegger* 1955) with IgG subclass specific antisera (*Oxelius* 1974) Ferritin conjugated IgG fraction of rabbit anti-human IgG was purchased from Cappel (Downington U.S.A lot No 6769) Serum from healthy rabbits not injected with streptococci or other antigens was used in some experiments (herinafter called normal rabbit serum) Rabbit IgG was obtained from normal rabbit serum as described by *Steinbuch & Audran* (1969) One isolated human IgG myeloma protein and the rabbit IgG were labelled with I (*McConahey & Dixon* 1966)

Determination of the Uptake of IgG by Streptococci

The uptake of ^{125}I labelled IgG myeloma protein by 0 2 ml standard suspension of streptococci was measured as described before and was expressed in per cent of 1 µg IgG added (*Christensen & Oxelius* 1974)

Trypsin Treatment of Streptococci

Trypsin treatment was performed essentially as described earlier (*Christensen & Oxelius* 1974) the streptococci were incubated at 37 C 1 hour with trypsin (Sigma) 1 mg per ml standard suspension of streptococci.

Preparation of Streptococci for Electron Microscopy Electron Microscopic Studies

Glutaraldehyde has been used for fixation of staphylococci for visualization of protein A (*Ackerson et al.* 1970) staphylococcal protein A reacts with the Fc part of IgG (*Forsgren & Sjöquist* 1966) To investigate the influence of glutaraldehyde on the interaction between streptococci and IgG not involving the antibody combining sites, the following introductory experiment was performed ^{125}I labelled IgG myeloma protein, 1 µg was added to 0 2 ml group A, type M 1 streptococcal standard suspension. After washings in PBS, the uptake of IgG was 70 per cent. Suspension of the streptococci in glutaraldehyde 15 mg/ml followed by washings in PBS did not diminish the uptake However if the streptococci were at first suspended in glutaraldehyde 15 mg/ml whereafter ^{125}I labelled IgG was added, the uptake on the streptococci of IgG was blocked.

The following standard preparation of the streptococci was performed for electron microscopic studies 1 ml standard suspension of streptococci was heated at 56 C, 30 min to kill the streptococci 0.5 ml IgG myeloma protein, 0.5 mg/ml (250 µg) PBS was added and the suspension left at 22 C for 1 hour followed by washings and suspension of the streptococci in PBS again. The streptococci were then incubated with ferritin conjugated IgG fraction of rabbit anti human IgG, 0 01 ml at 37 C for 1 hour fixed by addition of glutaraldehyde (final concentration 15 mg/ml) for 30 min and washed again. In some experiments, 0.5 ml rabbit serum was added instead of human IgG myeloma protein other experiments were performed with ferritin conjugated rabbit anti-human IgG antibodies only or without myeloma protein or anti human IgG antibodies present. The following procedure was slightly modified from *Ryter & Kellenberger* (1958) to the streptococcal pellets was added 1 ml of 1 per cent osmic acid in barbiturate-acetate buffer (pH 6 1) for prefixation and the streptococci were then centrifuged at 10 000 *g* for 10 min. The pellet was enrobed in agar (3 per cent in barbiturate acetate buffer) at 50 C. After hardening agar blocks of about 1 mm³ were cut and fixed at room temperature for 18 hours in osmic acid solution (10 part of 1 per cent osmic acid in Michaelis veronal acetate buffer (pH 6.1) and 1 part of 1 per cent Bacto tryptone solution containing 0 5 per cent NaCl) The blocks were washed 3 times, 10 min in Michaelis veronal-acetate buffer and treated for 2 hours with 1 per cent barbiturate buffered uranyl acetate (pH 6.1) at room temperature. Finally they were dehydrated with alcohol and propylene oxide and embedded in Epon (*Luft* 1961) The proportion between Epon components A and B was 1 9 After polymerisation during 6 days, the Epon-embedded material was cut with a

diamond knife (du Pont) mounted on a LKB-Ultratome III microtome.

All sections were examined with a Philips EM 300 electron microscope, at 60 kV with the use of 20 or 30 µm objective aperture. The micrographs were recorded on Kodak Fine Grain Release Positive (FGRP) film at a primary magnification of 3,000 × (Figs. 2, 3, 5, 6) or 10,000 × (Figs. 1 and 4). The film was developed with Kodak D 19-B developer.

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Electron Microscopic Studies

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The uptake of rabbit anti-human IgG antibodies (ferritin conjugated) was negligible after previous addition of rabbit serum instead of human IgG myeloma protein. The uptake of ferritin labelled rabbit anti human IgG antibodies on the surface of M 1 (M+ve) streptococci is shown in Fig. 6 (no human IgG myeloma protein added); the localization of ferritin is similar to that shown in Fig. 1.

The ferritin was also localized on the surface of type M 1 (M+ve) streptococci treated with IgG myeloma protein and anti-human IgG (ferritin conjugated) after previous treatment of the streptococci with trypsin (trypsin treatment diminishes the uptake of ^{125}I labelled IgG myeloma protein (*Christensen & Oxelius* 1974)).

TABLE 1. *The Uptake of I Labelled IgG Myeloma Protein by Some Group A Streptococci*

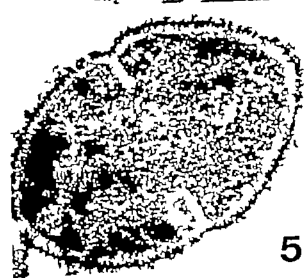
	Streptococcal strains							
	M 1		M 3			M 12		M 56
	M+	M	M+	M− R+	M+ e, R+	M+ve	M−v	M+
The uptake of ^{125}I labelled IgG myeloma protein (per cent of 1 µg IgG added) by 0.2 ml streptococcal standard suspension	70	10	13	16	12	11	14	20




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DISCUSSION

The streptococci, pretreated with human IgG myeloma protein took up ferritin labelled rabbit anti-human IgG antibodies on the surface of the cell walls. The same localization of the ferritin on the streptococci was obtained by allowing the rabbit antibodies to react with the streptococci directly by means of the interaction between rabbit IgG and streptococci, irrespective of the antibody combining sites (see below) i.e. by addition of ferritin labelled rabbit anti-human IgG antibodies without addition of human IgG myeloma protein. The uptake of ^{125}I labelled normal rabbit IgG by 0.2 ml M 1 (M+ve) streptococcal standard suspension diminished from 63 per cent to 2 per cent (of 1 µg IgG added) by addition of unlabelled human IgG myeloma protein. It thus seems reasonable to accept that the presence of ferritin labelled rabbit anti-human IgG antibodies on the surface of the streptococci, pretreated with human IgG myeloma protein, was caused by a reaction between the rabbit anti-human IgG antibodies and the IgG myeloma protein. On the other hand, it could be possible that the ferritin labelled rabbit anti-human IgG preparation might contain antibodies to streptococcal antigens obtained by natural immunization of the rabbit with streptococci. However it is not likely that the rabbit anti-human IgG preparation should contain antibodies to streptococcal antigens, capable to give a uniform staining of the outer protein layer of the antigenic heterogenous types of streptococci (group A carbohydrate antigen, like mucopeptide, are localized in the cell wall (see *Barkulis* (1968) for review))

By the use of purified IgG myeloma proteins, the probability of participation of the antibody combining sites in the reaction was minimized. The fact that the same picture was obtained by the use of four different IgG myeloma proteins supports the idea that the uptake of human IgG labelled with ferritin conjugated rabbit anti-human IgG antibodies was independent of the specificity of the antibody combining sites. Hence, the experiments showed, that the reaction between streptococci and the Fc part of the IgG was localized on the surface of the streptococcal cell wall. The septa (cross wall formations) of intact streptococcal cells, which were allowed to react with IgG myeloma protein and ferritin labelled rabbit anti-human IgG antibodies were not covered with ferritin. It might be possible that the septa could react with IgG myeloma proteins if sections of streptococci were allowed to react with IgG myeloma protein, but such experiments were not performed.

Attempts to quantitate the uptake of ferritin labelled rabbit anti-human IgG antibodies on the surface of the streptococci pretreated with human IgG myeloma proteins were not done. It is, however, notable that the streptococci, which took up a low amount of ^{125}I labelled IgG myeloma protein showed the same localization of ferritin labelled rabbit anti-human IgG antibodies when pretreated with IgG myeloma protein, as the strains with high uptake (compare for example M 3 (M+ve R+ve) (Fig. 5) and M 1 (M+ve) (Fig. 1))

Unlabelled rabbit IgG inhibited the uptake

Fig. 1. Streptococcus group A, type M 1 (M+ve) treated with human IgG myeloma protein and ferritin labelled rabbit anti-human IgG antibodies. Magnification × 80,000.

Fig. 2. Streptococcus group A, type M 1 (M+) No human IgG or ferritin labelled rabbit anti-human IgG antibodies added. Magnification × 80,000.

Fig. 3. Streptococcus group A, type M 3 (M+) treated with human IgG myeloma protein and ferritin labelled rabbit anti-human IgG antibodies. Magnification × 80,000.

Fig. 4. Streptococcus group A, type M 3 (M– R ve) treated with human IgG myeloma protein and ferritin labelled rabbit anti-human IgG antibodies. Magnification × 80,000.

Fig. 5. Streptococcus group A, type M 3 (M+ R ve) treated with human IgG myeloma protein and ferritin labelled rabbit anti-human IgG antibodies. Magnification × 80,000

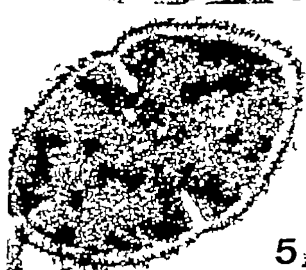
Fig. 6. Streptococcus group A, type M 1 (M+) treated with rabbit anti-human IgG antibodies (ferritin labelled); no human IgG myeloma protein added. Magnification × 80,000. Bar indicates 0.1 µm.




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for immunoglobulin G. J Immunol. *111* 1401–1406, 1973.

Lind, I Identification of *Neisseria gonorrhoeae* by means of fluorescent antibody technique. Acta path. microbiol. scand. *70* 613–629 1967

Luft J H Improvements in epoxy embedding methods. J Biophys. Biochem. Cytol. *9* 409–414 1961

McConahey P & Dixon, F J. A method of trace iodination of proteins for immunologic studies. Int. Arch. Allergy 29 185–189 1966

Ackerman D S White J G K on all, G Williams J R. C. & Quie P G Indirect visualization of *Staphylococcus aureus* protein A. J exp. Med *131* 1039–1047 1970

Oxelius V-A Chronic infections in family with hereditary deficiency of IgG 2 and IgG 4 Clin. and Exp. Immunol. *17* 19–27 1974

Ryter A. & Kellenberger E.. Étude au microscope électronique de plasmas contenant de l'acide désoxyribonucléique. Avec une contribution de *A Birch-Andersen* et *O Maaløe* Z. Naturforsch. *13 b* 597–600 1958

Scheidegger J J. Une micro-methode de l'immunoélectrophorèse. Internat. Arch. Allergy 7 103–110 1955

Steinbuch M & Audran R. The isolation of IgG from mammalian sera with the aid of caprylic acid. Arch. Biochem. Biophys. *134* 279–284 1969

Zabriskie J B. Mimetic relationships between group A streptococci and mammalian tissues. Adv Immunol. 7 147–188, 1967

of ^{125}I labelled IgG myeloma protein on the streptococci indicating a reaction between rabbit IgG and the streptococci independent from the antibody combining sites. *Cole* (1968) used ferritin labelled rabbit anti M protein antibodies in an attempt to localize the M protein on the surface of the streptococci because the streptococci are able to react with rabbit IgG independent of the specificity of the antibody combining sites, one can not be sure that the method *Cole* used really demonstrated the M protein in fact, a similar localization of ferritin conjugated rabbit anti human IgG antibodies was found in our experiments as in the work of *Cole*

It also follows from the present investigation and earlier works (*Christensen & Kronvall* 1974 *Christensen & Oxelius* 1974 and 1975 *Christensen et al* 1975 *Christensen* 1975) that cross reactions between streptococci and human tissues could be simulated by the capacity of the streptococci to react with IgG without involving the antibody combining sites In fact the capacity of group A C and G streptococci to be stained by FITC-labelled preimmune rabbit IgG has been described before (*Danielsson* 1965 *Jones & Foster* 1966 *Lind* 1967) Several cross reactions between streptococci and human tissues have been described these numerous studies have been comprehensively reviewed (*Zabriskie* 1967 *Kaplan* 1969 a b) None of these studies took into account the capacity of streptococci to react with IgG irrespective of the antibody combining sites.

The experiments showed that type M 1 (M+ve) streptococci took up more ^{125}I labelled IgG myeloma protein than the M−ve variant while essentially no difference was seen between such variants of type M 3 or type M 12. The relation between M protein and the capacity of the streptococci to take up ^{125}I labelled IgG myeloma protein will be studied further

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We acknowledge Mrs. *Ann Margret Carlsson* Mrs. *Maj-Lis Svensson* and Mrs. *Ulla Wulf* for their technical assistance

REFERENCES

Barkulis S S Chemical and enzymatic studies on the structure and composition of group A streptococcal cell walls. In Caravano, R. (Ed.) Current research on group A streptococcus. Excerpta Medica Foundation. Amsterdam, New York, London, Paris, Milan, Tokyo, Buenos Aires, 1968 p. 43–50

Christensen P Agglutinability of some selected streptococci by immune complexes. Acta path. microbiol scand. Sect. C, *83* 28–34 1975.

Christensen P & Kronvall G Capacity of group A B C, D and G streptococci to agglutinate sensitized sheep red cells. Acta path. microbiol. scand. Sect. B *82* 19–24 1974

Christensen P & Oxelius V A Quantitation of the uptake of human IgG by some streptococci groups A, B C and G Acta path. microbiol scand. Sect. B *82* 475–483 1974

Christensen P & Oxelius V A A reaction between some streptococci and IgA myeloma proteins. Acta path. microbiol. scand. Sect. C, *83* 184–188, 1975

Christensen P Oxelius V A & Hoier Madsen, M The mechanism of the L-agglutination of streptococci by sera from patients with rheumatoid arthritis. Acta path microbiol. scand. Sect. C, *83* 370–376 1975

Cole R M The structure of the group A streptococcal cell and its L-form. In Caravano, R. (Ed.) Current research on group A streptococcus. Excerpta Medica Foundation. Amsterdam New York, London Paris, Milan, Buenos Aires 1968 p 5–42.

Danielsson D The demonstration of *N gonorrhoeae* with the aid of fluorescent antibodies. 5 A comparison of different techniques-absorption, one step inhibition and counter staining for elimination of cross reactions. Acta derm. venereol *45* 61–73 1965

Forsgren A & Sjöquist J Protein A from S *aureus* I Pseudoimmune reaction with human γ-globulin. J Immunol. *97* 822–827 1966

Jones W & Foster I Papain-treated globulins in specific and cross-reacting immunofluorescent staining J Bact. *91* 984–986 1966

Kaplan M H Autoimmunity to heart and its relation to heart diseases. Progr Allergy *13* 408–439 1969 a.

Kaplan M H & Frengley J D Autoimmunity to heart in cardiac disease. Current concepts of the relation of autoimmunity to rheumatic fever postcardiotomy and postinfarction syndromes and cardiomyopathies. Am J Cardiol *24* 459 473 1969 b.

Kronvall G A surface component in group A, C and G streptococci with non-immune reactivity

HLA D unidentical, unrelated persons and 2) MLCs between HLA D identical HLA-A and B unidentical unrelated persons.

MATERIALS AND METHODS

HLA A and B typing was performed by the lymphocytotoxic micromethod described by *Kissmeyer-Nielsen & Kjerby* (6) and by the platelet complement fixation test (*Colombani et al.* (2). In few cases, family studies were included, allowing haplotype identification.

HLA-D typing was carried out on an earlier occasion using HLA A, B and D homozygous cells from children of first cousin marriages as stimulator cells in one way MLCs. Cells not responding to, or giving only weak response to, homozygous stimulator cells were assumed to carry the HLA-D determinant expressed by the homozygous stimulator cells (*Jørgensen et al.* 3 4 5) I our experiments, cells from HLA-D heterozygous unrelated individuals were used. The HLA D antigens involved were those earlier described as "S" and "L (5) but presently described as HLA-Dw2 and Dw4

MLC technique A macro-technique (1 ml cultures) based on the micromethod applied by *Jørgensen et al.* (3) was adopted for MIF production as described in detail earlier (7) Each experiment included two-way cultures, unmixed control cultures, one-way cultures in which one cell compartment was treated with mitomycin C, autologous control cultures, cultures where both cell compartments were treated with mitomycin, and unmixed control cultures of mitomycin treated cells.

In each MLC experiment cells from two (or more) *HLA A and B* or *HLA-D* identical unrelated persons and control cells from a person being HLA-A, B and D unidentical were included. The macro cultures were terminated after 72 hours of incubation and the cell free supernatants were kept at -20 C until assayed for MIF activity

MLC results were expressed by the stimulation ratio (*SR*) = the ratio between counts *per minute* (cpm) of a given two way culture (A+B) and the mean of cpm of the corresponding single cell cultures

$$\frac{(A) + (B)}{2}$$

MIF assay was performed in agarose medium as described by *Clausen* (1) and modified by us (7) Each supernatant was tested on cells from 2-4 random male blood donors.

The results were expressed as migration index (MI) = the ratio between the migratory area obtained by a given two way MLC supernatant (*A+B*) and the mean of the migratory area obtained by the supernatants from the corresponding single cell control cultures (A) and (B) This ratio was multiplied by 100 to obtain the MI. Based on the variation in size of replicate control areas only MIs below 85 were considered evidential of MIF activity

TABLE 1 *HLA A and B Type Stimulation Ratio (SR) and MIs of Serially Diluted Supernatants Recorded from MLCs with 11 Pairs of HLA A and B Identical Cells*

MLC No	Initials	HLA A and B type of reactants	SR cpm (A+B) / [cpm (A) + (B)] / 2	MI of supernatant dilutions 1:2 1:64‡					
				2	4	8	16	32	64
1	P+B*	A1 B8	1 4	113	103	105	98	101	103
2	JU/B*	A1 B8	1.9	94	94	104	111	100	100
3	JU HJ	A1 B8	1.9	93	127	90	106	110	/
4	G H	A1 A11 Bw25, B8	14 4	80	97	94	91	91	93
5	G E	A1 A11 Bw35 B8	30.8	50	96	87	89	89	101
6	G I	A1 A11 Bw35 B8	24.8	37	72	91	88	91	90
7	I O	A1 A11 Bw35 B8	27.2	30	56	71	80	89	96
8	G O	A1 A11 Bw35, B8	34.5	34	65	76	75	96	92
9	E O	A1 A11 Bw35, B8	29.2	33	63	77	83	105	93
10	E I	A1 A11 Bw35, B8	1 4	99	94	107	92	115	110
11	JL M	A2, A9 B8 B12	23 7	47	48	70	68	75	68

The person P and JU were HLA identical siblings, the other persons tested were unrelated.

‡ Supernatants were tested on cells from two male blood donors. The mean of all experiments is listed.

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THE IMPACT OF HLA-A, B OR HLA-D IDENTITY ON THE MIF PRODUCTION IN MLC

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Stenbjerg, S Mygind, K. & Jorgensen, F The impact of HLA-A, B or HLA D identity on the MIF production in MLC. Acta path. microbiol scand. Sect. C, 84 210–214 1976.

The relative influence on MIF release in MLC of different parts of the HLA region was studied in 1) MLCs between cells from HLA A and B identical (probably HLA D unidentical) unrelated persons and 2) MLCs between cells from HLA D identical, HLA A and B unidentical unrelated persons. It was found that the HLA D part of the region has the major effect on the MIF production. Studies of MIF activity in supernatants from mitomycin treated cultures lent some evidence to the hypothesis that there is an extreme linkage disequilibrium between the HLA D alleles and hypothetical separate MIF alleles Examination of families with cross-over between the HLA A, B and HLA D part of the region is required for confirmation of these findings.

Key words HLA-A HLA B HLA D MIF production MLC

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Recently we published data (7) indicating that 1) Mixed leucocyte cultures (MLC) between cells from HLA A and B identical siblings do not release leucocyte migration inhibition factor (MIF) during a 72 hour period and 2) one haplotype identical related combinations produce approximately one fourth of the amount of MIF found in super natants from cultures involving cells from two haplotype different siblings or unrelated per sons.

It was deduced that the degree of histocompatibility within the HLA region was reflected by the quantity of MIF released in MLC However the preliminary results did not allow conclusions concerning the part of the HLA region which had the major influence on MIF production in MLC. Further information in this regard could possibly be obtained from a study of MLCs involving cells being partially identical at the HLA region. Family studies must be assumed to be of less value in this context since recombination within the region is relatively rare with the consequence that HLA A and B identity as a rule is associated to HLA D identity We therefore decided to investigate MLCs between 1) HLA A and B identical, probably

The HLA nomenclature adopted in this work that recommended by the WHO-IUIS Terminology Comittee to be published in Histocompatibility Testing 1975 (Ed. F *Kissmeyer Nielsen* Munksgaard, Copenhagen 1975)

RESULTS

MIF assays were performed on supernatants from MLCs between cells from 11 HLA A and B identical unrelated combinations (Table 1) Four of these generated no MIF activity during a 72-hour culture period (exp. Nos. 1 2, 3, and 10) while most of the remaining combinations released substantial amounts of inhibitory substance MLC No. 4 released only slight amounts of MIF which were demonstrable only to a dilution of 1:2. The MIF negative combinations were found in combination with low SRs not differing from the patterns observed with HLA identical siblings (7) While SRs of a magnitude previously observed using cells from HLA A and B unidentical, unrelated persons (7) were recorded in MIF positive experiments. At an SR of 14.4 MLC experiment No. 4 showed intermediate stimulation with similar MIF release The observed pattern could be explained by co-incidental HLA-D identity in experiments Nos. 1 2, 3 and 10 HLA D haploidentity in experiment No. 4 and HLA D unidentity in the remaining combinations. Postexperimental HLA D typing of this series was not attempted.

A second series of MLCs employed 12 combinations of cells from six HLA-D identical unrelated persons (Table 2) The SRs ranged from 3.7 19.5 i.e. from almost non-stimulation to a magnitude usually recorded from haploidentical related combinations. The MIF quantities were well correlated to the degree of activation as measured by the SR (the product moment correlation coefficient, = 0.93) With the exception of MLC No 14 MIF was demonstrable only when the SR exceeded 10. The largest amounts of MIF were found in MLCs with "haploidentical" response In several cases, the cells shared one (or two) HLA-A or B determinants which apparently did not influence the MIF production.

DISCUSSION

It is the aim of the present study to investigate the relative influence of different parts of the HLA region on the MIF release in MLC. Earlier we found (7) that MIF activity cannot be demonstrated in MLCs between HLA identical siblings while increasing amounts are produced in MLCs involving 1) HLA A and B haploidentical siblings and 2) HLA A and B unidentical siblings or random unrelated persons, in that order

The present results indicate that MLCs performed with HLA-A and B identical cells from unrelated persons generate large amounts of MIF except in cases of MLC negativity indicating coincidental HLA D identity HLA D identity alone seems to ensure low grade or missing MIF production in correlation with the mitotic activity recorded by the SR. Thus the major influence on MIF response lies within the HLA D parts of the HLA region

Whether MIF is generated solely as a result of HLA-D disparity or whether separate MIF determinants are involved remains to be solved. It may be speculated that the mitotic activity induced by minor HLA-D disparity may obscure the influence of a hypothetical separate MIF determinant. If so, further information might arise from the study of MIF activity in MLCs employing HLA D identical cells with blocked DNA synthesis (mitomycin cultures ($A_m + B_m$))

Usually mitomycin blocked cell cultures release some MIF activity when composed of cells from HLA D unidentical persons (7) The cell combinations listed in Table 2 were studied in mitomycin cultures, and in no case did the supernatants contain any demonstrable MIF activity (unpublished data) This finding may be due to insensitivity of the test system or it may be speculated that some MIF activity might emerge after longer culture periods. If taken at face value, these results could indicate the presence of a separate MIF locus in extreme linkage disequilibrium with the HLA D locus. Studies of cells from families with appropriate chromosomal recombinations are required for verification of such speculations. However at present the overall results seem to point strongly to the

TABLE 2. *HLA D Type HLA A and B Type Stimulation Ratio (SR) and MIs of Serially Diluted Supernatants Recorded from MLCs with 12 Pairs of HLA D Identical Cells*

MLC No.	Initials	HLA D type	HLA A and B type	$\frac{\text{cpm (AB)}}{\frac{\text{cpm (A)} + \text{(B)}}{2}}$	SR	MI of dilution§ 1 2	1 4	1 8	1 16	1 32	1 64
12	M + B	w2/w4	A3 Aw19 Bw40 – A1 A2 B7 B12	1576/155	10.1	85	97	96	106	102	95
13	B + I*	w2/w4	A3 Aw19 Bw40 – Aw32 Bw37/A2, Bw15	667/119	5 6	98	105	101	106	105	98
14	B + E*	w2/w4	A3 Aw19 Bw40 – Aw32 Bw37 /A2 Bw15	825/175	4.5	86	95	103	103	97	98
15	M + I	w2/w4	A1 A2 B7 B12 – Aw32 Bw37/A2, Bw15	1547/117	13.2	76	78	92	91	84	93
16	M + E	w2/w4	A1 A2 B7 B12 – Aw32 Bw37/A2 Bw15	2200/121	18.2	78	94	77	87	93	88
17	Ø + I	w2/w4	A2 A3 B7 – Aw32 Bw37/A2, Bw15	1396/137	10.2	77	82	89	87	97	/
18	Ø + G	w2/w4	A2 A3 B7 – A3 A11,B7 Bw22	1300/136	9 6	84	96	96	98	96	102
19	G + B	w2/w4	A3 A11 B7 Bw22 – A3 Aw19 Bw40	472/129	3 7	110	116	98	89	95	95
20	G + M	w2/w4	A3 A11 B7 Bw22 – A1 A2 B7 B12	971/124	7.8	101	98	104	109	98	98
21	G + E	w2/w4	A3 A11 B7 Bw22 – Aw32 Bw37/A2, Bw15	695/154	4.5	96	90	97	103	98	105
22	Ø + E	w2/w4	A2 A3 B7 – Aw32 Bw37/A2 Bw15	2928/151	19.3	65	83	83	83	96	90
23	Ø + M	w2/w4	A2, A3 B7 – A1 A2 B7 B12	2871/180	15.9	81	91	90	100	96	97

* The persons I and E were HLA identical siblings, the other persons tested were unrelated.

§ Supernatants were tested on cells from 2 male blood donors. The mean of all experiments is listed.

RESULTS

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conclusion that HLA D disparity serves as the signal for MIF release in MLC.

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REFERENCES

1 *Clausen J E.* Migration inhibitory effect of cell free supernatants from mixed human lymphocyte cultures. J Immunol *108* 455–459 1972.

2. *Colombani J D Amaro J Gabb B. Smith G & Svejgaard A* International agreement on a microtechnique of platelet complement fixation (Pl C Fix) Transpl. Proc. *3* 121–126 1971

3 *Jørgensen F Lamm L. U & Kissmeyer Nielsen F* Mixed lymphocyte cultures with inbred individuals. Tissue Antigens *3* 323-339 1973

4 *Jørgensen F., Lamm L. U & Kissmeyer-Nielsen F* Three LD (MLC) determinants A Danish population study Tissue Antigens 4 419–428 1974

5 *Jørgensen F., Lamm L. U & Kissmeyer-Nielsen F* MLC results among unrelated can be predicted. Tissue Antigens 5 262 263, 1975

6 *Kissmeyer Nielsen F & Kjerbye K. E.* Lymphocytotoxic microtechnique. Purification of lymphocytes by flotation. In Curtoni, E. S Mattiuz, P L. & Tosi, R. M. (Eds) Histocompatibility Testing Munksgaard, Copenhagen *1967* pp. *381–383.*

7 *Stenbjerg S & Mygind K* Migration inhibitory factor (MIF) and the human major histocompatibility complex (MHC) Acta path. microbiol scand. Sect. C, *83* 157 164 1975

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ANTINUCLEAR FACTORS IN SERA FROM HEALTHY BLOOD DONORS

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Wiik, A. Antinuclear factors in sera from healthy blood donors. Acta path. microbiol. scand. Sect. C, 84 215–220, 1976.

Sera obtained from 466 healthy blood donors were investigated for presence of granulocyte specific and organ-nonspecific antinuclear factors of the IgM, IgA and IgG classes, 110 sera also for complement-fixing and IgD class antinuclear factors. When undiluted sera were studied, most sera were found to contain antinuclear factors of one or more immunoglobulin classes (77 per cent) IgM IgA and IgG antinuclear factors were found in 53 44 and 14 per cent, respectively At dilution 1 16, antinuclear factors of the IgM class were still detected in 16 per cent of the sera, while IgA and IgG antinuclear factors were demonstrated in 6 and 2 per cent, respectively Organ-nonspecific antinuclear factors were clearly more common than granulocyte-specific antinuclear factors No serum contained complement-fixing or IgD class antinuclear factors This study indicates the importance of using qualitative as well as quantitative techniques to distinguish between antinuclear factors in health and disease

Key words Antinuclear factors sera healthy blood donors.

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For theoretical reasons it is important to know whether or not auto-antibodies should be considered abnormal in the sense that they are produced in chronic inflammatory diseases only From a practical viewpoint it is necessary to know the prevalence of auto-antibodies in normal control persons to be able to interprete the findings in patients.

Many studies have dealt with the occurrence of antinuclear factors (ANF) in different states of disease but very limited work has been done to show ANF in normal persons. It is commonly believed that ANF production is due to some disturbance in immunological tolerance mechanisms to self antigens, a situation mainly encountered in chronic auto-immune and inflammatory diseases. However it has been established that ANF may be detected in a number of healthy individuals in whom signs of auto-immune diseases or chronic infections are absent (1 3 5 7 12 14 19 22)

When we started to study normal blood donor sera for the presence of organ-non-specific and granulocyte-specific ANF (ON ANF and GS-ANF) using a polyspecific anti-human immunoglobulin (Ig) conjugate we were struck by the many positive results obtained. Accordingly we decided to study the Ig class nature and the complement-fixability of these ANF

TABLE 1 *The Age and Sex Distribution of the Donors (No.)*

Sex	20–29 years	30–39 years	40–49 years	50–59 years	Total
♀	43	22	33	23	121
♂	121	95	81	48	345
♀ + ♂	164	117	114	71	466

MATERIALS AND METHODS

Sera were obtained from 466 randomly selected consecutive healthy blood donors from the blood bank at Bispebjerg Hospital, Copenhagen. All the donors had subscribed a written statement denying a previous or recent presence of any venereal diseases, hepatitis or chronic diseases. It may be appropriate to mention that blood donors in Denmark have no financial interest in blood donation since they are not paid for their service. The age and sex distribution of the donors is shown in Table 1. Sera were stored at –20 C. ANF tests were run currently within a few weeks after serum sampling.

GS-ANF and ON ANF were demonstrated by indirect immunofluorescence technique employing human thyroid tissue sections and human leucocytes as nuclear substrates (19, 20, 21). Fluorescein isothiocyanate-labelled rabbit IgG specific for human γ, μ and α chains as well as the β_1c-component of human complement were purchased from Dakopatts, Copenhagen. All details regarding the technique, the characteristics of the conjugates and their specificity as tested by immunoelectrophoresis and by direct immunofluorescence have been reported earlier (19, 20, 21). A δ chain specific fluorescein isothiocyanate conjugate was purchased from Behringwerke, Marburg, Germany. Also this conjugate showed a specific reaction in tests on monoclonal bone marrow specimens. Phosphate buffered saline pH 7.2 (PBS) served as diluent for the serum and conjugate dilutions. All sera were screened for IgM, IgA and IgG ANF undiluted and diluted 1:16. One hundred and ten undiluted sera were also screened for presence of IgD ANF and complement-C3 fixing ANF.

RESULTS

Seventy seven per cent of the undiluted sera showed presence of ANF of one Ig class or more. Fig. 1 depicts the prevalence of ON ANF and GS-ANF of the IgM, IgA and IgG classes. The majority of the undiluted serum samples contained IgM ANF, but also IgA and IgG ANF were commonly detected in the undiluted specimens. The antibodies were mostly of the ON ANF variety. At dilution

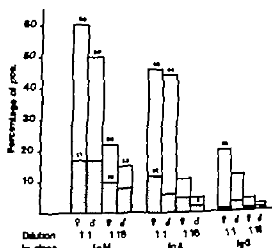


Fig. 1. Prevalence of IgM, IgA and IgG granulocyte-specific and organ-nonspecific antinuclear factors in sera from healthy blood donors. ☐ GS-ANF ☐ ON ANF

1:16 a considerable percentage of the sera still showed presence of IgM and IgA ANF whereas IgG ANF were rarely detected.

The prevalence of IgM ANF demonstrated in undiluted sera was about the same in the different age and sex groups except in the group of women between 40 and 50 years in whom the prevalence was higher (Fig. 2). It appeared from studies of diluted sera, that women exhibited clear differences ascribable to age, while men between 50 and 60 years showed a higher prevalence than men in the other age groups due to the more frequent finding of IgM GS-ANF.

The prevalence of IgA ANF detectable both in undiluted and diluted specimens seemed to increase steadily with age (Fig. 3) and the antibodies were mainly of the ON ANF variety in all groups.

In men, the prevalence of IgG ANF both in undiluted and diluted sera was rather constant at all ages (Fig. 4). Undiluted sera from

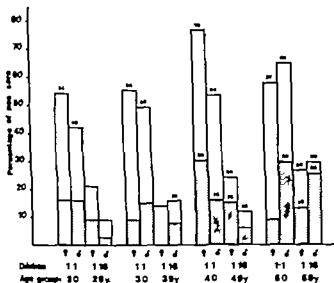


Fig. 2 Prevalence of IgM granulocyte-specific and organ-nonspecific antinuclear factors in sera from healthy blood donors of different ages. ☐ GS-ANF ☐ ON-ANF

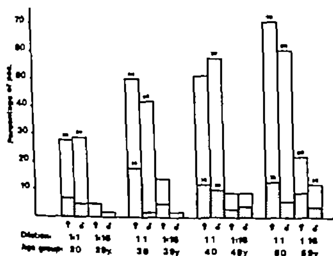


Fig. 3 Prevalence of IgA granulocyte-specific and organ-nonspecific antinuclear factors in sera from healthy blood donors of different ages. ☐ GS-ANF ☐ ON-ANF

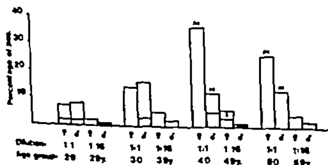


Fig. 4 Prevalence of IgG granulocyte-specific and organ-nonspecific antinuclear factors in sera from healthy blood donors of different ages. ☐ GS-ANF ☐ ON ANF

women showed a peak *prevalence in the 40 to 50* year decade, whereas such an increase in prevalence was *not seen in the diluted sera*.

Prozone phenomena were not seen in any case.

One hundred and ten consecutive undiluted sera were studied for presence of IgD ANF but such antibodies were not found in any of the sera The same sera were studied for complement-C3 fixing properties of ANF All sera showed negative reactions

DISCUSSION

Several studies have shown that ANF may be present in some sera *from healthy blood donors* (3 5 7) The prevalence is mostly reported to be low in normal control persons (5 7) but some authors have demonstrated ANF in a high percentage of these (3 12) Factors which may explain these discrepancies are mainly of technical nature (11 19) while some may be related to the age and sex distribution of the persons studied, since ANF are encountered more frequently in women and in elderly people (1 3 14 19 22)

The details of the technique used in this study have been discussed earlier and it is sufficient to emphasize that the sensitivity is high due to the use of well-defined and highly specific conjugates and a fluorescence microscope *yielding close to optimal conditions for* the reading of the results (19) The use of *two different nuclear substrates* allowed identification of two specificities of ANF ON ANF and GS-ANF (4 19 20 21)

By this technique, we have previously demonstrated a high prevalence of ANF in healthy control persons around 60 to 90 years old (19) We found IgG IgA and IgM ANF in a titre of $\geq$ 16 in 20 36 and 60 per cent, respectively

The present study shows a high prevalence of IgM and IgA ANF in undiluted sera from young healthy persons, while *IgG ANF* were seen less frequently Even when diluted 1 16 about one fifth *of the sera were found to* contain detectable IgM ANF about 6 per cent IgA ANF and *about 2 per* cent IgG ANF *ANF of all Ig* classes were a little more common in women than in men. In undiluted serum specimens both from women and men, an age dependent increase in prevalence of *IgA ANF* was seen, and women aged 40 to 50 years exhibited a higher prevalence of IgM *and IgG ANF than females* in the other age groups. In males, a trend towards an age dependent *increase in ANF content* was found as regards IgM ANF and the same applies to both sexes as *regards IgA* ANF IgG ANF titres seemed to be independent of age and sex.

It is well known that certain infections and medications may give rise to ANF production in healthy individuals (6 8 9 10) It is also commonly anticipated that *ANF* frequently may be found in healthy relatives of patients suffering from systemic lupus erythematosus and rheumatoid arthritis (2 11) However such factors cannot explain the high prevalence of ANF observed in this investigation. It is more likely that small concentrations of ANF primarily of the IgM and IgA classes, are normal constituents of serum. These antibodies most probably serve a physiological function in connection with the removal of nuclear debris constantly produced in the organism when cells die.

It seems rather unlikely that unwanted reactions between *Ig aggregates in the* donor sera and/or conjugates and Fc receptors on *granulocytes, monocytes and lymphocytes* account for the results obtained the reasons being as follows 1 Storage time at −20 C was short and sera were not heat inactivated. 2. IgM in which case Fc receptors have not been described, was the predominant Ig class of ANF 3 ON-ANF reactions with blood cells were confirmed by the positive staining of thyreoid nuclei. 4 Repeated investigation of some sera after several months of storage gave identical results. 5 Daily control tests using the conjugates alone consistently showed negative reactions with nuclei of both nuclear substrates.

From a practical point of view it is essential to know that the discovery of ANF in serum of patients should not be regarded a pathological finding unless they exceed a cer

tain titre of IgG class ANF in persons aged 20 to 60 years. Essentially indentical data have been obtained by investigation of 66 sera derived from persons between 0 and 20 years (16)

ANF in normal control sera never possessed complement fixing properties in agreement with the predominance of IgM and IgA antibodies (15 18) The lack of complement fixability may imply a negligible phlogogenicity of these antibodies.

IgD ANF were never found in the normal donor sera, thus supporting the impression that IgD ANF mainly occur in rheumatic diseases such as systemic lupus erythematosus and rheumatoid arthritis (13 17 20) in the latter primarily in connection with pronounced serological activity (17)

In accordance with earlier studies, ON ANF were more common than GS-ANF in healthy controls (19)

In conclusion, the present study indicates that ANF may be regarded as normal serum constituents probably serving a physiological purpose. Qualitative as well as quantitative measures must be taken into account when these ANT are to be distinguished from those encountered in pathological conditions. This fact calls for caution when results from different laboratories are compared, and stresses the importance of bringing more uniformity into the methodology used in ANF serology

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REFERENCES

1 *Cammarata R. J Rodnan G P & Fennel R H* Serum anti-gammaglobulin and antinuclear factors in the aged J Amer Med Ass. *199* 455–458, 1967

2 Dalaka *T G MacS en R N M Whaley K., Dick W C., Boyle J A., Jasani, M K., Wilson M E., Buchanan W W & Goudie R. B.*, Conjugal prevalence of rheumatoid arthritis, rheumatoid factor and other autoantibodies in rheumatoid arthritis. Clin. exp. Immunol. *3* 761–769 1968.

3. *Doniach, D., Roitt I & Taylor K B.* Autoimmune phenomena in pernicious anaemia. Brit. Med. J *1* 1374–1379 1963.

4 *Faber V & Elling, P.*, Leucocyte-specific antinuclear factors in patients with Felty's syndrome, rheumatoid arthritis, systemic lupus erythematosus and other diseases. Acta Med. Scand. *179* 257 267 1966

5 *Faber V & Elling, P.*, Antinuclear factors as determined by the immunofluorescent antibody technique. Acta Med. Scand. *177* 309–319 1965

6. *Feltkamp T E W., Dorhout M es E. J & Nieuwenhuis M G* Autoantibodies related to treatment with chlorthalidone and α-methyldopa. Acta Med. Scand. *187* 219–223 1970.

7 *Hasker J., Mackay I R. & Miller J J.* The incidence of antinuclear factor in human disease Australas. Ann. Med. *14* 96–101 1965

8. *Kaplan M E. & Tan E. M* Antinuclear antibodies in infectious mononucleosis. Lancet *I*. 561–563 1968.

9 *Klajman A., Camin Belsky N., Kimchi, A. & Ben-Efraim S.*, Occurrence, immunoglobulin pattern and specificity of antinuclear antibodies in sera of procaine amide treated patients. Clin. exp Immunol. *7* 641–649 1970

10 *Perry H M., Chaplin H Carmody S., Haynes C & Frei C* Immunologic findings in patients receiving methyldopa: A prospective study J Lab. clin. Med. *78* 905–917 1971

11 *Pollak V E.* Antinuclear antibodies in families of patients with systemic lupus erythematosus. New Engl. J Med. 271 165–171 1964

12. *Ritchie R F* The clinical significance of titered antinuclear antibodies. Arthritis Rheum. *10* 544–552, 1967

13 *Ritchie R F* IgD antinuclear antibodies. Arthritis Rheum. *11* 506, 1968 (abstr.)

14 *S ec K. H & V est R. C* Age-related antinuclear factors Immunologic characteristics and associated clinical aspects. Arthritis Rheum. *10* 509–516 1967

15 *T jo T & Fries G J.*, Lupus nephritis Varying complement-fixing properties of immunoglobulin G antibodies to antigens of cell nuclei. Science *161* 904 906, 1968.

16. *Wiik A.* Unpublished work.

17 *Wiik A.*, Incidence and possible significance of IgD granulocyte-specific antinuclear factors in neutropenic and non-neutropenic cases of rheumatoid arthritis. Scand. J. Rheumatol., Suppl. 8, abstract 13–13 1975

18 *Wiik A* Circulating immune complexes involving granulocyte-specific antinuclear factors in Felty's syndrome and rheumatoid arthritis. Acta path microbiol. scand Sect. C, *83* 354–364 1975
19 *Wiik A Jensen E & Friis J* Granulocyte-specific antinuclear factors in synovial fluids and sera from patients with rheumatoid arthritis. Ann. rheum. Dis. *33* 515–522 1974
20 *Wiik A & Munthe E* Restrictions among heavy and light chain determinants of granulocyte-specific antinuclear factors. Immunology *23*: 53–60 1972
21 *Wiik A & Munthe E.* Complement-fixing granulocyte-specific antinuclear factors in neutropenic cases of rheumatoid arthritis. Immunology *26* 1127–1134 1974
22 *Wilkens R F Whitaker R. R., Anderson R V & Beroen D* Significance of antinuclear factors in older persons. Ann. rheum. Dis. *26* 306–310 1967

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RESTITUTION OF STREPTOZOTOCIN INDUCED DIABETES MELLITUS IN NUDE MICE WITH PANCREATIC GRAFTS FROM THE RAT

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Buschard, K. & Rygaard, J. Restitution of streptozotocin induced diabetes mellitus in nude mice with pancreatic grafts from the rat. Acta path. microbiol. scand. Sect. C, 84 221-226, 1976.

Transplants of 1-3 rat pancreases have proven to restitute streptozotocin induced diabetes in athymic nude mice. One out of 9 diabetic nude mice showed restitution following subcutaneous implantation of 1 rat pancreas. Three out of 6 nude mice were restituted by 2-3 grafts. Implants of rat submandibular gland did not restitute nude mice similarly treated with streptozotocin. The model seems of interest in studies of inter-species transplantation of pancreas.

Key words: Streptozotocin diabetes rat pancreatic grafts nude mouse heterotransplantation.

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Nude mice with streptozotocin induced diabetes have been restituted by transplantation of rat pancreas.

Many recent studies have concerned the transplantation of total pancreas or isolated islet tissue in the mouse (7) rat (1 4) and hamster (5 6) after induction of diabetes by streptozotocin or alloxan. The effect of such transplants on established diabetes is variable. By contrast with these previous studies in which donors and recipients were always of the same inbred strain, this present study concerns heterologous transplants. Total pancreas preparations from neonatal Hooded Rats were transplanted to the nude mouse. The nude mouse displays thymic agenesis (9) and, in consequence the animal lacks any cell-mediated immune response. This is evidenced by a number of transplantation studies of allogeneic and heterogeneic tissues (12) which are readily accepted by nude mouse recipients.

MATERIALS AND METHODS

The recipient nudes, both male and female, were 6-8 week old outcome of a tenth to eleventh back cross mating with BALB/c mice. Breeding was conducted at the Pathological Anatomical Institute, Kommunehospitalet, Copenhagen (12).

Diabetes was induced with streptozotocin (11) (Upjohn, Kalamazoo, Michigan) diluted in saline and given intravenously in the tail within 4 minutes of preparation. Total dosage was 220 mg/kg BW. Blood samples were taken by orbital puncture. 50 µl aliquots were incubated with orthotoluidin reagent (art. 8300, Merck, Darmstadt, BRD) and blood sugar was determined spectrophotometrically. The blood samples were taken from not fasting animals. The upper limit of

normal blood sugar was taken to be 180 mg/100 ml, under which value glucosuria has never been observed. Urine samples were analyzed with Tes-Tape® (Lilly Indianapolis Ind.)

Only those nude mice in which blood sugar determinations, repeated at an interval of 5–7 days indicated certain diabetes were used as recipients.

The donors were 2 3 day old Hooded Rats. The whole pancreas was removed under sterile conditions immediately after decapitation of the donors, and 1–3 such preparations were implanted subcutaneously in the flank of the recipient through a dorsal incision. Recipients were kept under light propanidid anesthesia (Epontol® Bayer Leverkusen BRD)

In control of organ specific effects, eight diabetic nude mice received subcutaneous transplants of two submandibular glands from 2–3 day old Hooded Rats.

In control that immune responses were unaffected five not streptozotocin treated nude mice were transplanted with rat pancreas just as the study animals proper

Following transplantation all recipients were weighed weekly and urine and/or blood samples were analyzed. Animals were daily observed. Twelve animals died spontaneously and the remainder (16 of 28) were sacrificed in terminal condition or at the end of experiment. Tissues were taken and prepared for histological study. Pancreas, and pancreas and submandibular gland transplants, were fixed in Bouin's fluid, embedded in paraffin wax, and 5 μm sections were cut and stained with hematoxylin and eosine, and aldehyde fuchsin, before light microscopy study

RESULTS

Clinical observations and measurements pointed the animals in which there was restitution of the diabetic state. Weight was stable or increased, and appearances and activity were normal for the mutant. Survival was approximately the same as in not treated nude mice. Not restituted mice displayed rapid weight loss, progressive deterioration of

TABLE 1 *Effect of One Rat Pancreas Transplant in Diabetic Nude Mice*

Mouse nr	Before transplant. BS mg/100 ml	Before transplant. US +/—	Survival days	Following transplant. BS mg/100 ml	Following transplant. US +/—	Weight change g	Restitution of diabetes
1	308	+	34	553	+	–4 4	No
2	582	+	-	45_	+	–2.2	No
3	225	+	23	153	—	+0.8	Yes
4	425	+	10	n.d.	+	–2.4	No
5	332	+	23	n.d.	+	–3.5	No
6	300	+	10	416	+	–0.5	No
7	456	+	1	n.d.	+	–4 0	No
8	361	+	29	656	+	–4 7	No
9	264	+		n.d.	+	–1.3	No

BS = blood sugar US = urinary sugar n.d. = not done.
All mice except nr 4 were histologically studied.

TABLE 2 *Effect of Two-Three Rat Pancreas Transplants in Diabetic Nude Mice*

Mouse nr	Before transplant. BS mg/100 ml	Before transplant. US +/—	Survival days	Following transplant. BS mg/100 ml	Following transplant. US +/—	Weight change g	Restitution of diabetes
1	421	+	14	n.d.	+	–2.8	No
2	361	+	39	176	—	+3 4	Yes
3	216	+	27	97	—	+0 7	Yes
4	228	+	14	n d.	+	–0 1	No
5	305	+	93	116	—	+4.8	Yes
6	300	+	21	383	+	+0.5	No

All mice except nrs. 2 and 4 histologically studied

TABLE 3. *Effect of Two Rat Submandibular Gland Transplants in Diabetic Nude Mice*

Mouse nr	Before transplant. BS mg/100 ml	Before transplant. US +/—	Survival days	Following transplant. BS mg/100 ml	Following transplant. US +/—	Weight change g	Restitution of diabetes
1	527	+	3	n d.	n d.	n.d.	–
2	209	+	16	335	+	–3.6	No
3	587	+	8	n.d.	n d.	n.d.	–
4	344	+	17	471	+	–4.6	No
5	495	+	22	447	+	–6.1	No
6	277	+	3	n.d.	n.d.	n.d.	–
7	514	+	9	437	+	–4.8	No
8	382	+	5	n d.	n.d.	n d.	–

Mice nrs. 4 and 7 subsequent histological studies. The remainder not so studied.

general condition, and were inactive. Mortality is recorded in Tables 1–3 and mentioned in the following report of the results in each group of study animals

Diabetic Nude Mice
with One Pancreas Transplant (Table 1)

Of nine animals, glucosuria resolved after two weeks in one (no. 3) and blood sugar levels fell to within the normal range. This animal gained in weight and thrived till the 23rd day when it was sacrificed, and the histological studies were made. The remaining eight animals were persistently diabetic one died on the tenth day the others were sacrificed between days 7 and 34

Diabetic Nude Mice
with 2 3 Pancreas Transplants (Table 2)

In three of 6 mice with 2 3 rat pancreas transplants, glucosuria resolved and blood sugar fell to within the normal range between the first and the second week after transplantation. These animals gained in weight and survived from 27 to 93 days. The remaining three animals died from days 14 to 21

Diabetic Nude Mice
with Submandibular Gland Grafts (Table 3)

Eight animals continued to display diabetes. Six died between days 3 and 22 two were sacrificed on days 9 and 17 respectively

Not Streptozotocin Treated Nude Mice
with Two Rat Pancreas Transplants

In five animals of this group there was no significant change in blood sugar levels, and no animal displayed glucosuria. Three died at 34 days with clinical wasting syndrome, and the remaining two were sacrificed at the same time.

Morphological Examinations

Macroscopically no differences were observed between the pancreases of streptozotocin treated and not streptozotocin treated mice. Microscopically no changes were observed in exocrine pancreatic tissue, but some cells in the Islets of Langerhans displayed vacuolization of the cytoplasm, and varying degrees of nuclear pyknosis, when animals had received streptozotocin and became diabetic. Streptozotocin treated mice which did not become diabetic, and not treated mice displayed no such pancreatic changes.

Macroscopically the pancreas grafts were seen to be of the same size as at transplantation, occasionally somewhat smaller

Histologically all transplants were healed in, encapsulated in a richly vascular net of fibrous connective tissue (Fig. 1) Acinar exocrine structures were mildly or markedly dilated and there was flattening of acinar epithelium. In some larger grafts central necrosis was observed. In restituted mice Islets of Langerhans, seen in varying numbers,

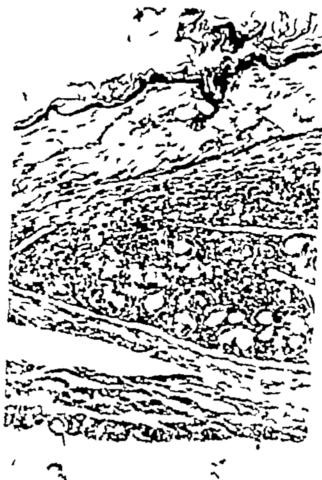


Fig. 1. Rat pancreas transplant in the subcutaneous space of nude mouse recipient. Fibroblastic proliferation with granulocytic and histiocytic infiltration. H & E.

were virtually always of normal histological appearance (Figs. 2 and 3).

The cellular reaction to the grafts was more pronounced than reactions to normal allogeneic or heterogeneic skin and human malignant tumours (12), probably due to an irritative effect of the product of the exocrine element. Granulocytes and histiocytes were dominant. No lymphocyte infiltration, suggestive of rejection, was seen.

DISCUSSION

As recorded, restitution of mice with streptozotocin induced diabetes by transplantation of heterologous whole pancreas grafts from neonatal rats was achieved. Previous similar studies differ in that pancreas grafts have always derived from congeneic donors.

A prerequisite of the reported experiments was that recipients should be nude mice. The degree of success depends on the congenital absence of the thymus in the nude, or rather on the consequent absence of cell-mediated immune responses. It was an important consideration that serum insulin level in the nude mouse is as in mice in general, also found for other hormones in comparative studies of nude and normal mice (8).

Streptozotocin was used to induce diabetes, because spontaneous remission has never been reported in other mice so treated. *Rerup & Tarding* (11) report that all normal albino NMRI mice became diabetic after streptozotocin administration in dosage of 200 mg/kg BW, but 12 of 60 nude mice so treated did not become diabetic. Eight normal inbred BALB/c mice, the background strain of the nude mice, all developed diabetes after streptozotocin administration in the same dose. This suggests that there may be a secondary immune reaction implicated in streptozotocin induction of diabetes. The phenomenon is presently being investigated.

Streptozotocin mortality was somewhat higher than otherwise reported (11), in that eighteen streptozotocin treated nude mice died from diabetes within one week of treatment, before having an organ graft. This is well accounted for by the immune defect in the nude.

To avoid leaking of exocrine secretions at the transplantation site, some authors have undertaken ligation of the pancreatic duct 6–8 weeks before removing the pancreas for transplantation (7). Islet tissue remains intact. This could be thought advantageous, but it is known that exocrine function have some effect on Islet hormone production (3) and the issue is thus not clear. Fragmented pancreas transplants have also been used (4, 7).

We transplanted whole pancreas without interference. No immune rejection phenomena were observed, and we interpret the central necrosis as a simple expression of in-

Fig 2 Islets of Langerhans (bottom, center) and dilated acini of exocrine pancreatic tissue (top, left) in connective tissue Recipient reinfused. H & E



Fig 3 Islet tissue in same graft as in Fig 2, Aldehyde Fuchsin Stain.

[illegible]

adequate or too slow revascularization and diffusion

Sites chosen for implantation vary The testis and the anterior chamber of the eye support grafts but diabetes persists (2 4) Some endocrine effect has also been observed after pancreas transplant under the renal capsule (1) and to the cheek pouch of the hamster (5) Many have employed subcutaneous transplantation (2 6 7) which is *simple and allows* multiple transplantations.

Those nude mice which responded evidenced restitution within two weeks. Other studies, where transplantation was preceded by pancreatic duct ligation report delays of about five weeks (7)

Histologically nude mouse graft acceptance was total as has also been reported when human foetal pancreas was transplanted (10) The only deviation from normal histological finding was some dilatation of acini In restituted nude recipients, exuberant Islet tissue was preserved but only remnants enmeshed in fibrous connective tissue were seen in not restituted animals. Signs of inflammation were probably due to an irritative effect of pancreatic exocrine secretion. Streptozotocin induced diabetic nude mouse pancreas always evidenced mild necrosis with disruption of Islet cells which displayed vacuolization of the cytoplasm and nuclear pyknosis. Such changes were not seen in graft Islet tissue.

Multiple transplantation did not lead to hypoglycaemia as judged by our weekly measurements of blood sugar values. This suggests that beta-cells retain some regulatory mechanisms even following interspecies transplantation. This particular phenomenon and also the implication which arises from this study that an immune reaction may be implicated in Islet tissue destruction suggests that the nude mouse may be an interesting and valuable object *in diabetes research.*

Exploitation of the nude mouse in investigation of human pancreas transplantation would seem likely to be rewarding.

REFERENCES

1 *Brown J Molnar I G Clark W & Mella, Y* Control of experimental diabetes mellitus in rats by transplantation of fetal pancreas Science *184* 1377–1379 1974

2. *Browning, H & Resnik P* Homologous and heterologous transplantation of pancreatic tissue in normal and diabetic mice. Yale J Biol Med. *24* 141–152 1951–52.

3 *Goberna R Fussgänger R. D Raptis S Telib M & Pfeiffer E F.* The role of the exocrine pancreas in the stimulation of insulin secretion by intestinal hormones. *Diabetologia* 7 68–72 1971

4 *Gonet A E & Renold A E.* Homografting of fetal rat pancreas. Diabetologia *1* 91 96, 1965

5 *House E L. Jacobs M S & Pansky B* Homoplastic transplantation of pancreas in diabetic hamsters. Transplant. Bull. *28* 435–441 1961

6 *House E. L., Pansky B & Jacobs, M S.* The effect of pancreatic homografts on the diabetic state of alloxan treated hamsters Anat Rec. *140* 341–344 1961

7 *Kramp R C., Congdon C C & Smith, L H* Recovery from experimental diabetes mellitus in mice after pancreas transplantation Diabetes *23* 183–188, 1974

8. *Ohsawa N Matsuzaki F Etoh A. & Nomura T* Endocrine functions of the nude mouse. In *Rygaard J & Povlsen, C O* (Eds.) Proc. I Internat. Workshop on nude mice, G Fischer Verlag Stuttgart, 1974 p. 221–226

9 *Pantelouris E M* Absence of thymus in a mouse mutant. Nature *217* 370–371 1968.

10 *Povlsen C O Skakkebæk N Rygaard J & Jensen G* Heterotransplantation of human foetal organs to the mouse mutant nude. Nature *248* 247 249 1974

11 *Rerup J & Tarding F* Streptozotocin- and alloxandiabetes in mice. *Eur J Pharmacol.* 7 89–96 1969

12 *Rygaard J* Thymus & Self Immunobiology of the mouse mutant nude. F.A.D.L., Copenhagen, and *J Wiley & Sons,* London, 1973

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A LEUCOCYTE MIGRATION STUDY ON THE CELL MEDIATED IMMUNITY AGAINST ADULT HUMAN ORAL MUCOSA AND STREPTOCOCCAL ANTIGENS IN PATIENTS WITH RECURRENT APHTHOUS STOMATITIS

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Donatsky, O. A leucocyte migration study on the cell-mediated immunity against adult human oral mucosa and streptococcal antigens in patients with recurrent aphthous stomatitis. Acta path. microbiol. scand. Sect. C, 84: 227-234, 1976.

In vitro demonstration of cell-mediated immunity (CMI) against adult human oral mucosa antigen (AHOM) and streptococcal antigens was accomplished by means of the leucocyte migration test (LMT) in patients with recurrent aphthous stomatitis (RAS). If antigenic extracts of AHOM, *Streptococcus sanguis* strain 2A or *Streptococcus pyogenes* strain M5 were used in the LMT experiments, the distribution of migration indices (MI) over patients with RAS differed significantly from the distribution over the controls. In patients with other oral lesions, these MI-distributions did not differ significantly from those among the controls. Extracts of other tissues such as kidney and uterus tissues or of other bacterial antigens such as *Brucella abortus* and *Escherichia coli* did not reveal any significant differences between the LMT responses in RAS and controls. The present findings show that CMI against AHOM antigen and streptococcal antigens are features characteristic of RAS. Furthermore, the results indicate that common antigenic determinants might be shared by AHOM and streptococci.

Key words: Recurrent aphthous stomatitis; cell-mediated immunity; leucocyte migration test.

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Several immunological investigations have demonstrated that auto-immunity against oral mucosa antigen and streptococcal hypersensitivity are characteristic features of recurrent aphthous stomatitis (RAS) (3, 4, 5, 7, 8, 9, 10, 12, 14, 15, 19, 20, 21). Significantly elevated antibody-titres against foetal and adult human oral mucosa antigen and against *Streptococcus sanguis* strain 2A (1) indicating humoral mediated immunity (HMI) have been demonstrated in patients with RAS (7, 9, 10, 14).

Cell mediated immunity (CMI) against an extract of foetal oral mucosa was primarily demonstrated in a lymphocyte-transforma

tion study by *Lehner* (15). The lymphocytes from patients with RAS showed significantly increased transformation determined by their uptake of ^{14}C thymidine. Lymphocytotoxicity against epithelial cells from adult human gingiva or oral mucosa was demonstrated by help of target cell destruction tests where the final viability of the oral epithelial target cells was determined by exclusion of trypan blue dye at the end of the incubation period (4, 5, 19).

Cell mediated streptococcal immunity was primarily indicated by the fact that intracutaneous injection of Strep. 2A was able to induce a delayed type hypersensitivity reaction in some patients with RAS (12). A similar effect in patients with RAS was demonstrated using a suspension of Bencard's streptococcal antigens (20).

Furthermore, cell mediated *in vitro* hypersensitivity against Strep. 2A has been demonstrated in a preliminary study of leucocyte migration by *Donatsky & Bendixen* (8). However, the streptococcal hypersensitivity theory was not confirmed by *Francis & Oppenheim* (11). These authors found impaired lymphocyte stimulation by Strep. 2A and suggested a deficiency of cell mediated immunity against Strep. 2A in RAS.

The purpose of the present investigation was, by means of the leucocyte migration test (LMT), to study the cell mediated immunity (CMI) against adult human oral mucosa antigen and against streptococcal antigens in patients with RAS.

MATERIAL AND METHODS

The below features were observed in four groups of persons.

Group 1 (Fig. 1)

Twenty three persons (13 females, age 19–60, average 38; 10 males, age 21–60, average 35) without anamnestic or clinical evidence of RAS or other oral mucosal diseases.

Eighteen persons (11 females, age 16–75, average 39; 7 males, age 21–52, average 31) without any anamnestic or clinical evidence of RAS or other mucosal diseases. These 18 persons had each a tooth extracted or surgically removed one week before the experiments. At the same time a punch biopsy (3–5 mm in diameter) was taken from normal appearing oral mucosa in the same region.

Nine patients (3 females, age 31–71, average 49; 6 males, age 30–58, average 41) with oral lichen planus.

Six patients (5 females, age 35–83, average 54; 1 male, age 78) with discoid lupus erythematosus and one patient (female, age 71) with systemic lupus erythematosus. All the patients with lupus erythematosus showed oral mucosal lesions.

Twenty-six patients (15 females, age 17–71, average 36; 11 males, age 19–60, average 32) with anamnestically and clinically characteristic RAS. In 13 patients RAS was in an active period with ulcers, in 13 it was in an inactive period.

All the above 83 adult persons were tested against adult human oral mucosa antigen (AHOM).

Group 2 (Fig. 2)

Twenty-four persons (15 females, age 20–63, average 43; 9 males, age 19–44, average 28) without any anamnestic or clinical evidence of RAS or other oral mucosal diseases.

Furthermore, the above 18 patients with oral mucosal wounds, the 9 patients with oral lichen planus, and the 7 patients with oral lupus erythematosus were included in the series presented in Fig. 2.

Twenty-five patients (19 females, age 22–77, average 46; 6 males, age 23–39, average 32) with anamnestically and clinically characteristic RAS. In eight patients it was in an active period, in 17 in an inactive period. All the above 83 persons were tested against Strep. 2A antigen.

Group 3 (Fig. 3)

Sixteen persons (11 females, age 22–56, average 39; 5 males, age 23–44, average 32) without any anamnestic or clinical evidence of RAS or other oral diseases. Furthermore, the above 34 patients with oral mucosal wounds, oral lichen planus, and oral lupus erythematosus were included in the series presented in Fig. 3.

Thirteen patients (10 females, age 17–77, average 41; 3 males, age 23–39, average 37) with anamnestically and clinically characteristic RAS. In 6 patients it was in an active period, in 7 in an inactive period.

All the 63 persons above were tested against Strep. M5.

Group 4 (Fig. 4)

In the experiments presented in Fig. 4 groups of control persons without any anamnestic or clinical evidence of RAS or other oral mucosal diseases and groups of patients with anamnestically and

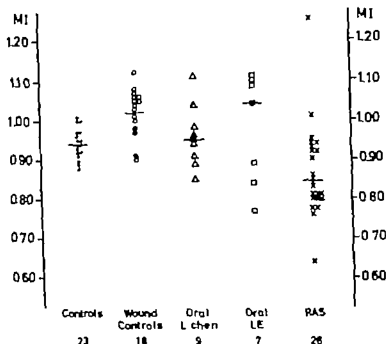


Fig 1 Leucocyte migration test with adult human oral mucosa (250 μg protein per ml) showing the migration indices (MI) of 23 normal controls, 18 wound controls, 9 patients with oral lichen, 7 patients with oral lupus erythematosus (Oral LE) and 26 patients with recurrent aphthous stomatitis (RAS)

clinically characteristic RAS were tested against brucella, *E coli* and tissue extracts from kidney and uterus, respectively

Fourteen control (7 females, age 19–55 average 36 7 males, age 22 55 average 36) and 15 patients with RAS (5 females, age 18–41 average 31 8 males, age 18 58, average 31) were tested against brucella antigens

Fourteen control (9 females, age 20–60 average 35 5 males, age 20–60, average 36) and 22 patients with RAS (15 females, age 17 77 average 43 7 males, age 19–59 average 30) were tested against *E coli* antigens

Fifteen controls (9 females, age 19 55 average 37 6 males, age 22 44 average 31) and 15 patients with RAS 6 females, age 23 41 average 32 9 males, age 18 58, average 30) were tested against kidney antigens

Eighteen control (10 females, age 19 55 average 37 8 males age 22 55 average 33) and 15 patients with RAS (6 females, age 23 41 average 32 9 males, age 18 58 average 30) were tested against uterus antigens

The leucocyte migration technique (LMT) was performed according to the description by *Søborg & Bendixen* (22) and *Bendixen & Søborg* (2) as modified according to *Maini et al* (18). Peripheral blood was collected from cubital vein into 10 ml polysterol tubes containing 250 I.u. of heparin and 2 0 ml of 5 per cent Dextran 2.0 The tubes were carefully inverted 10 times and placed for 1 hour at 37 C. After sedimentation of the erythrocytes, the supernatants were transferred to polyethylene tubes and centrifugalized at 1000 × g for 5 minutes. The leucocytes were washed three times in Hanks balanced salt solution and resuspended in TC 199 with 10 per cent horse serum to concentration of 2.2 × 10⁸ cells per ml. The leucocytes were then collected into capillary tubes (Internal diameter 0.6 mm) The capillary tubes were sealed at one end by melting and centrifugalized at 3.000 r p m. for 10 minutes. The tubes were then cut just below the cell-fluid interphase and placed in 0.5 ml culture chambers containing TC 199 and 10 per cent horse serum. After addition of the wanted volume of antigen extract, the chambers were sealed with microscope cover slips. Three culture chambers were set up for each concentration of the antigen employed. The cell migration from the open end of the capillary tube was measured in projection microscope after 24 hours of incubation at 37 C. The average migration area of a series of antigen containing cultures (Ma) and the average migration area of a series of control cultures (Mc) were used for calculation of the migration index (MI) in the following way

tion study by *Lehner* (15) The lymphocytes from patients with RAS showed significantly increased transformation, determined by their uptake of ^{14}C thymidine. Lymphocytotoxicity against epithelial cells from adult human gingiva or oral mucosa was demonstrated by help of target cell destruction tests where the final viability of the oral epithelial target cells was determined by exclusion of trypan blue dye at the end of the incubation period (4 5 19)

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All the 63 persons above were tested against Strep M5

Group 4 (Fig 4)

In the experiments presented in Fig 4 groups of control persons without any anamnestic or clinical evidence of RAS or other oral mucosal diseases and groups of patients with anamnestically and

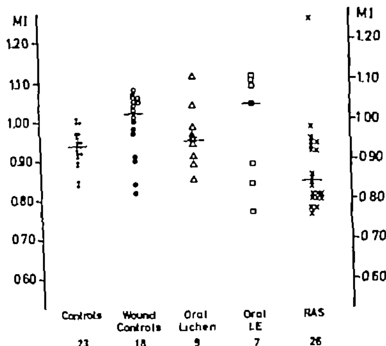


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Fifteen controls (9 females, age 19–55, average 37; 6 males, age 22–44, average 31) and 15 patients with RAS (6 females, age 23–41, average 32; 9 males, age 18–58, average 30) were tested against kidney antigens.

Eighteen controls (10 females, age 19–55, average 37; 8 males, age 22–55, average 35) and 15 patients with RAS (6 females, age 23–41, average 32; 9 males, age 18–58, average 30) were tested against uterus antigens.

The leucocyte migration technique (LMT) was performed according to the description by *Søborg & Bendixen* (22) and *Bendixen & Søborg* (2) as modified according to *Manni et al.* (18). Peripheral blood was collected from the cubital vein into 10 ml polystyrol tubes containing 250 [illegible] of heparin and 2.0 ml of 5 per cent Dextran 250. The tubes were carefully inverted 10 times and placed for 1 hour at 37 °C. After sedimentation of the erythrocytes, the supernatants were transferred to polyethylene tubes and centrifugalized at 1000 × g for 5 minutes. The leucocytes were washed three times in Hanks balanced salt solution and resuspended in TC 199 with 10 per cent horse serum to a concentration of 2.2×10^6 cells per ml. The leucocytes were then collected into capillary tubes (internal diameter 0.6 mm). The capillary tubes were sealed at one end by melting and centrifugalized at 3000 r.p.m. for 10 minutes. The tubes were then cut just below the cell-fluid interphase and placed in 0.5 ml culture chambers containing TC 199 and 10 per cent horse serum. After addition of the wanted volume of antigen extract, the chambers were sealed with microscope cover slips. Three culture chambers were set up for each concentration of the antigens employed. The cell migration from the open end of the capillary tube was measured in a projection microscope after 24 hours of incubation at 37 °C. The average migration area of a series of antigen containing cultures (Ma) and the average migration area of a series of control cultures (Mc) were used for calculation of the migration index (MI) in the following way

$MI = \frac{M}{M}$ A MI less than 1 0 therefore indicates an inhibition, and a MI greater than 1 0 a stimulation of the leucocyte migration.

Preparation of antigens The antigens used were tissue antigens extracted from normal adult human unkeratinized oral mucosa, foetal kidney and human uterus. Furthermore the following microbial antigens were used Strep. 2A, Strep M5 brucella, and *E coli*

The tissue antigen extracts were prepared by cutting the biopsies into small pieces which were subsequently suspended in Hank s solution and homogenized. The homogenates were kept at 4 C over night and then centrifugalized at 1000 × *g* for 20 minutes. The protein concentration of the supernatant was adjusted to 1 mg protein per ml by dilution with Hank s solution. The oral mucosa extract was stored at −20 C. The extracts from kidney and uterus were lyophilized.

The Strep 2A was a subculture from a strain of *Streptococcus sanguis* strain 2A. This strain was isolated from recurrent aphthous ulceration and appears to be related to or identical with *Streptococcus sanguis* group H, ATCC 10556 (1) The Strep. M5 was a subculture from a strain of haemolytic *Streptococcus pyogenes* group A, type M5 NCTC 100065 The streptococcal strains were cultured separately on solid media at 33 C for 24 hours, harvested and washed three times in distilled water Five grammes of each culture were then suspended in 25 ml distilled water and disintegrated by sonication for 3 × 45 s at 20000 Hz/s, using a NSE Mullard 60W 19 mm anode with 9.5 mm tip cooled with ice-water The disintegrates were ultracentrifugalized at 105000 × *g* for 1 hour at 4 C and the supernatants were stored at −20 C The strain of *Escherichia coli* was used as a whole cell suspension. The suspension was stored at −20 C. The brucella bacteria used were derived from a lyophilized strain of *Brucella abortus*

In preliminary LMT experiments, the highest non-toxic antigen concentrations were as follows.

adult human oral mucosa (AHOM)	250 µg protein per ml
foetal human kidney	100 µg protein per ml
human uterus	250 µg protein per ml
Strep. 2A	20 µg protein per ml
Strep M5	2 µg protein per ml
E. coli	1×10^{4} bacteria per ml
Brucella abortus	1×10^{7} bacteria per ml

The strain of Strep. 2A was kindly provided by Dr Graykowski, U.S.A. and the strains of Strep M5 and brucella by Statens Seruminstitut, Copenhagen, Denmark. The streptococcal antigen extracts were kindly prepared by Dr *N Høiby* and the strain of *E coli* by Dr *Gerd Hoff* (Blegdamshospitalet Copenhagen Denmark)

RESULTS

The results are presented in Fig 1 2 3 and 4 Fig 1 shows the distributions of MI in controls, in patients with other oral mucosal lesions (wound controls, oral lichen planus, and oral lupus erythematosus) and in patients with RAS Extracts of adult human oral mucosa (AHOM) was used as antigen in these experiments. The MI medians in the different samples are indicated and were 0 94 1 02 0 95 1 04 and 0 84 respectively Statistical analysis using the Mann-Whitney U test shows that the MI is significantly lower in the group of patients with RAS than in the group of controls ($p = 0\,0012$) The group of wound controls and the groups of patients with oral lichen planus or oral lupus erythematosus did not differ significantly from the group of controls ($p = 0\,0566$, $p = 0\,7529$ $p = 0\,6063$)

Fig 2 shows that the distribution of MI is similar to that seen when Strep. 2A is used as antigen. The MI medians were 0.97 0 99 0 91 0 99 and 0 81 respectively Statistical analysis (Mann Whitney U test) shows that the MI is significantly lower in the group of patients with RAS than in the group of controls ($p = 0\,0008$) The group of wound controls and the groups of patients with oral lichen planus or oral lupus erythematosus did not differ significantly from the group of controls ($p = 0\,5661$ $p = 0.4660$ $p = 0.3554$)

The results of the LMT using Strep M5 as antigen are shown in Fig 3 Distributions similar to those seen when AHOM or Strep. 2A were used as antigens are revealed. The

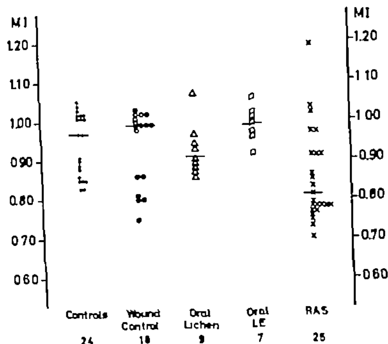


Fig. 2. Leucocyte migration test with Strep. 2A (20 μg protein per ml) showing the migration indices (MI) of 24 normal controls, 18 wound controls, 9 patients with oral lichen, 7 patients with oral lupus erythematosus (Oral LE) and 25 patients with recurrent phthous stomatitis (RAS)

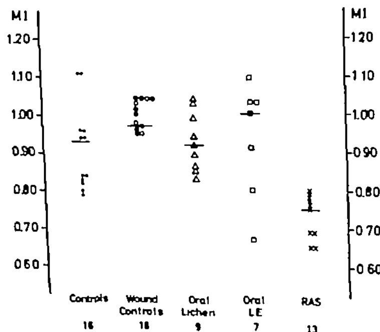


Fig. 3. Leucocyte migration test with Strep. M5 (2 μg protein per ml) showing the migration indices (MI) of 16 normal controls, 18 wound controls, 9 patients with oral lichen, 7 patients with oral lupus erythematosus (Oral LE) and 13 patients with [illegible] stomatitis (RAS)

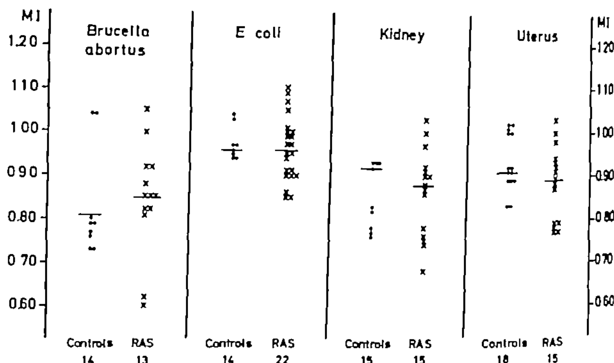


Fig 4 Leucocyte migration test with *Brucella abortus* (1×10^{7} bacteria per ml) *E. coli* (1×10^{4} bacteria per ml) kidney (100 µg protein per ml) and uterus (250 µg protein per ml) antigens showing the migration indices (MI) of normal controls and patients with recurrent aphthous stomatitis (RAS)

MI medians were 0 93 0 97 0 92 1 00 and 0 75 respectively Statistical analysis (Mann-Whitney U test) shows that the MI is significantly lower in the group of patients with RAS than in the group of controls ($p < 0.00005$) The group of wound controls and the groups of patient with oral lichen planus or oral lupus erythematosus did not differ significantly from the group of controls ($p > 0.10$)

Fig 4 shows the distribution of MI in different groups of controls and patients with RAS when *Brucella abortus* *E coli* kidney or uterus antigens were used in the LMT experiments None of these antigens revealed any significant differences between distributions of MI in the groups of controls and the corresponding groups of patients with RAS (Mann Whitney U test $p > 0.10$ $p > 0.10$ $p > 0.10$ $p > 0.10$) In the present series, the LMT responses against AHOM antigen Strep 2A and Strep M5 were not related to sex as the respective distributions of MI did not differ significantly when the groups of females were related to the groups of males (Mann Whitney U test $p > 0.10$) Furthermore, there was apparently no correlation between age and MI values in the above LMT-experiments (Spearman rank correlation coefficient, $r = -0.046$ 0 106 -0.211 respectively $p > 0.10$) The results of the investigation of a possible correlation between recurrence of RAS and MI will be presented separately

DISCUSSION

In the present study it was possible to demonstrate *in vitro* cell mediated immunity (CMI) against adult human oral mucosa antigen (AHOM) and against streptococcal antigens in patients with RAS These findings support the results obtained in other *in vitro* investigations into the CMI in RAS (4 8 15 19) and indicate that auto-immunity against oral mucosa as well as a streptococcal hypersensitivity might be involved in the pathogenesis of RAS

Previous studies have revealed that lymphocytes from patients with RAS are stim-

ulated, by contact with oral mucosal antigens, to release blastogenic and cytotoxic lymphokines (4 15 19). In the present experiments, the lymphocytes from patients with RAS stimulated by contact with AHOM, released a lymphokine migration inhibitory factor (MIF) as detected by the LMT

An extract of adult human oral mucosa (AHOM) was used as antigen in the present LMT. In previous studies using the lymphocyte transformation test or the lymphocytotoxicity test, the antigens used were derived from an extract of foetal human oral mucosa (FHOM) gingival target cells and in some cases adult human oral mucosal target cells (4 16, 19). The above results suggest that cross-reacting antigenic determinants are shared by FHOM AHOM and adult human gingival tissue. Cross-reaction between FHOM antigen and AHOM antigen has been demonstrated and discussed by *Donatsky* (7) and various studies of different groups of RAS concerning the HMI in RAS have revealed auto-antibodies both against FHOM and against AHOM (7 10 13 14 16)

Any significant difference in the distribution of MI in controls and patients with RAS was not shown when other tissue extracts such as extracts of kidney and uterus tissue were used as antigen in the LMT (Fig 4). Organ-specificity of the CMI is also indicated by the results of lymphocytotoxicity and lymphocyte transformation experiments (6 15. As far as the immunological investigations into the organ-specificity of the auto-antibodies in RAS are concerned, the results are varying. Absorption experiments have revealed cross-reaction between foetal human mucosa of the mouth, pharynx, larynx, oesophagus, conjunctiva, vagina, as well as colon and skin (17. In the same study the auto-antibodies against FHOM antigen were shown to be species unspecific. The last observation was supported in the immunofluorescence (IF) study by *Donatsky & Dabelsteen* (10. However in this IF-study no antibodies against adult human skin were detected

The observations illustrated in Fig. 2 showing a significant LMT response against Strep. 2A in RAS are in agreement with the *in vivo* observations by *Graykowski et al.* (12) and *Shore & Shelley* (21) and confirm the results obtained by *Donatsky & Bendixen* (8). These results are, as previously discussed (9) at a variance with the immunological hyporeactivity to Strep. 2A demonstrated in lymphocyte transformation experiments (11). Preliminary studies in relation to the present LMT experiments revealed that heat killed whole Strep. 2A bacteria were not equally convenient as the antigenic extract from sonicated Strep 2A. Therefore, a further explanation of the above discrepancy might be that the procedures used for the preparation of the Strep 2A antigen have been different.

The LMT results obtained by Strep. M5 *Brucella abortus* and *E. coli* (Fig 3 4) indicate that the LMT response against bacterial antigens might be streptococcal specific. This theory is further supported by the negative results of attempts to detect significantly raised antibody titres against neisseria and streptomyces in sera from patients with RAS (9). Nevertheless, further immunological investigations into the CMI and HMI against micro-organisms isolated from aphthous ulcerations are necessary before any final conclusions can be drawn concerning the specificity of the streptococcal hypersensitivity in RAS.

The present results showing CMI against Strep. 2A antigen and adult human oral mucosa antigen (AHOM) in RAS support the theory that cross-reacting streptococcal antigens and AHOM-antigens are involved in the pathogenesis of RAS (7 13)

The *in vitro* CMI against AHOM antigen or streptococcal antigens was not significantly different in the various groups of controls with other oral mucosal lesions (Figs. 1 2, 3). These findings are in agreement with the observations by *Lehner* (13 14 15) and *Rogers et al.* (19). In these studies, however the control groups comprised patients with varying oral mucosal lesions the present groups of patients with oral lichen planus and oral lupus erythematosus are too small to conclude whether CMI and/or HMI re

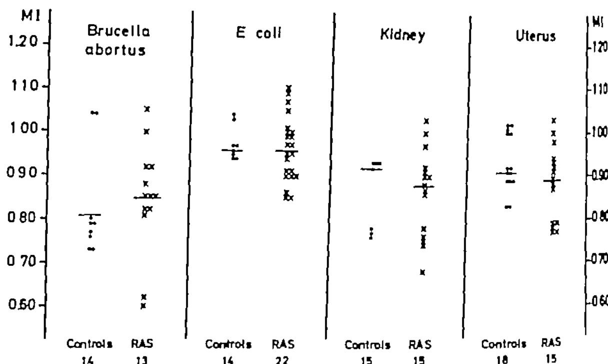


Fig 4 Leucocyte migration test with *Brucella abortus* (1×10^7 bacteria per ml) *E. coli* (1×10^8 bacteria per ml) kidney (100 μg protein per ml) and uterus (250 μg protein per ml) antigens showing the migration indices (MI) of normal controls and patients with recurrent aphthous stomatitis (RAS)

MI medians were 0 93 0 97 0.92 1 00 and 0 75 respectively Statistical analysis (Mann Whitney U test) shows that the MI is significantly lower in the group of patients with RAS than in the group of controls ($p < 0\ 00005$) The group of wound controls and the groups of patient with oral lichen planus or oral lupus erythematosus did not differ significantly from the group of controls ($p > 0\ 10$)

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STUDY OF SUBSTANCES RELATED TO CARCINOEMBRYONIC ANTIGENS CEA NCA AND ASSOCIATION WITH α_1 ANTICHYMOTRYPSIN

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Ørjasæter H. Study of substances related to carcinoembryonic antigens, CEA-NCA and association with -antichymotrypsin. Acta path. microbiol scand. Sect C, 84 235–244, 1976.

The -protein which w previously detected in PCA extracts of malignant tumours, normal tissues, sera and other body fluids has been further tested. The α-protein was identified by immunological methods as -antichymotrypsin Experiments by crossed immunoelectrophoresis showed that this protein cross-reacts with the β_E-protein found in the PCA extracts. The β_E-protein show heterogeneity in molecular size electrophoretic mobility and it has been demonstrated that it shares antigenic determinants with the CEA or β_1-molecule Cross-reaction between β_F and other glycoproteins found in the PCA extract of normal or malignant tissue or serum was not detected.

Key words Carcinoembryonic antigens CEA-NCA α -antichymotrypsin.

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In previous studies of carcino-embryonic antigens (CEA) we have described three antigenic substances in the perchloric acid (PCA) extract of colonic carcinomas (16, 17 One is the β_I-protein which was found to give reactions of complete identity with GOLD's CEA in gel diffusion studies The second was called β_F-protein It was found to share antigenic determinants with β and was therefore also referred to as *non specific cross-reacting antigen* or NCA (6, 17) The third substance found in the PCA extract had the electrophoretic mobility of an α protein. The α-protein was found together with the β_I- and β_F-proteins and could be detected in extracts of normal tissue serum and urine as well as in extracts of malignant tissue (17) Indications were found that the α protein was antigenically associated with the β_E-protein. Preliminary studies suggested that our α-protein was different from the *main third antigen* or MTA found in colonic carcinoma by *v Kleist & Burtin* (7) and *v Kleist et al.* (8)

This study was done to identify and further characterize the α-protein and to clarify its association with the β_I- and β_E-proteins or with other proteins found in the PCA extract of colonic carcinomas.

MATERIALS AND METHODS

Perchloric acid (PCA) extract of colonic adenocarcinomas was prepared as described (16). Normal human serum was extracted in a similar way by equal amounts of 1.2 M PCA. The extracts

sponses similar to those in RAS may be involved in other oral mucosal diseases.

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REFERENCES

1 *Barile M F Francis T C & Graykowski E. A Streptococcus sanguis* in the pathogenesis of recurrent apthous stomatitis. In Guze, L. B (Ed.) Microbial protoplasts, spheroplasts and L-forms. Williams & Wilkins 1968 pp. 444–456

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3 *Brody H A & Silverman S.*. Studies on recurrent oral aphtae I Clinical and laboratory comparisons. Oral Surg *27* 27–34 1969

4 *Dolby A E* Recurrent apthous ulceration. Effect of sera and peripheral blood lymphocytes upon oral epithelial tissue culture cells. Immunology *17* 709–714 1969

5 *Dolby A E.* Mikulicz's recurrent oral aphthae. The effect of anti-lymphocyte serum upon the in vitro cytotoxicity of lymphocytes from patients for oral epithelial cells. Clin Exp Immunol *7* 681–686 1970

6 *Dolby A E* The effect of lymphocytes from sufferers from recurrent apthous ulceration upon colon cells in tissue culture. Gut *13* 387–389 1972

7 *Donatsky O* An immunofluorescence study on the cross-reaction between Strep 2A and human oral mucosa. Scand. J Dent. Res. *83* 111–119 1975

8. *Donatsky O & Bendixen G* In vitro demonstration of cellular hypersensitivity to Strep 2A in recurrent apthous stomatitis by means of the leucocyte migration test. Acta allerg *27* 137–144 1972

9 *Donatsky O & Dabelsteen E.* An immunofluorescence study on the humoral immunity to Strep 2A in recurrent apthous stomatitis. Acta path. microbiol. scand Sect. B *82* 107–11? 1974a.

10 *Donatsky O & Dabelsteen E.*. An immunofluorescence study on the humoral immunity to adult human oral mucosa in recurrent apthous stomatitis. Acta allerg *29* 308-318, 1974b.

11 *Francis T C & Oppenheim J J* Impaired lymphocyte stimulation by some streptococcal antigens in patients with recurrent apthous stomatitis and rheumatic heart disease. Clin. Exp. Immunol. *6* 573–586 1970

12 *Graykowski E A Barile M F Lee W B & Stanley H R.* Recurrent apthous stomatitis. Clinical therapeutic, histopathologic and hypersensitivity aspects. J Amer med. Ass. *196* 637–644 1966

13 *Lehner T* Recurrent apthous ulceration and autoimmunity Lancet *2* 1134–1135 1964.

14 *Lehner T* Autoimmunity and management of recurrent oral ulceration. Brit. dent. J *122* 15–20 1967a.

15 *Lehner T.*. Stimulation of lymphocyte transformation by tissue homogenates in recurrent oral ulceration. Immunology *13* 159–166, 1967b.

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17 *Lehner T* Characterization of mucosal antibodies in recurrent apthous ulceration and Behcet's syndrome. Arch. oral Biol. *14* 843–853 1969b.

18 *Manns R N Rolfe L. M Magrath I T & Dumonde D C* Standardization of the leucocyte migration test. Int. Arch. Allergy *45* 308–321 1973

19 *Rogers R S Sams W M & Shorter R. G* Lymphocytotoxicity in recurrent apthous stomatitis. Lymphocytotoxicity for oral epithelial cells in recurrent apthous stomatitis and Behcet's syndrome. Arch. Derm. *109* 361 363 1974

20 *Sallay K Hajós M K & Bánóczy J* Untersuchungen über den allergischen Pathomechanismus der chronischrezidivierenden Aphten. Allergie u. Immunologie *17* 17–23 1971

21 *Shore R N & Shelley W B* Treatment of apthous stomatitis by suppression of intralesional streptococci Arch. Derm. *109* 400–402, 1974

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REFERENCES

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2 *Bendixen G & Soborg M* A leucocyte migration technique for in vitro detection of cellular (delayed type) hypersensitivity in man A survey Dan. med Bull *16* 1–6 1969

3 *Brody H A & Silverman S* Studies on recurrent oral aphtae. I Clinical and laboratory comparisons. Oral Surg *27* 27–34 1969

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13 *Lehner T* Recurrent apthous ulceration and autoimmunity Lancet *2* 1154–1155, 1964

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18. *Maini R N Roffe L M, Magrath I T & Dumonde D C* Standardization of the leucocyte migration test. Int. Arch. Allergy *45* 308–321 1973

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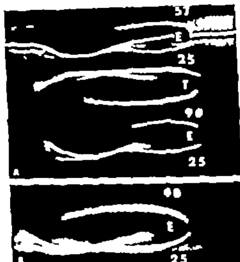


Fig 2. Demonstration of β_F-protein in PCA extract and Sephadex fractions, and characterization of antibody activity in antisera nos. 57 25 and 90. Immunoelectrophoresis in agarose gel.

Trough 57 Antiserum no. 57 absorbed with normal human serum and PCA extract of normal liver Activity against β_F or NCA. (α-protein present in the absorbed antiserum)

Trough 25 Antiserum no. 25 absorbed with normal human serum A Activity against β_F and α-protein (weak reaction against α_1-antitrypsin remains) B Reaction of complete identity with antiserum no. 90

Trough 90 Antiserum no. 90, absorbed with normal human serum Activity against β_F

Well E Pooled Sephadex G 200 fractions (Nos 50–70, Fig 8) prepared from PCA extract of liver metastases of colonic carcinoma.

Well T PCA extract of colonic carcinoma

Anode to the left. I B the electrophoresis was prolonged for 30 min and the precipitin lines augmented by tannic treatment Reaction of complete identity between the α-protein in well E and the protein present in the absorbed antiserum no. 57 is seen

detected by antiserum no. 57 (Fig 1 A, B) which defines the NCA determinants on β_F Reaction of identity was also observed when β_F-protein was tested against antisera nos 90 and 25 (Fig ? B

Antiserum no 90 reacts with determinants on β_F as well as on β_F-protein (Fig. 1 C) while antiserum no 25 shows no reaction with *different concentrations of β_F* (Fig. 1 D) thus lacking activity against the NCA determinants on this protein. *In Fig 1 C it* is seen that antiserum no 90 also reacts with β_F-specific determinants, indicated by spurring over the β_F-line.

The electrophoretic mobility of β_F-protein is shown in Fig 2. The elongated *β_F-line* demonstrates electrophoretic heterogeneity in *this protein, especially* in the unfractionated PCA extracts, T The Sephadex G 200 fractions show a more limited heterogeneity Antisera nos. 57 90 and 25 detect β_F-protein of identical electrophoretic mobility

In Fig 2 also the α protein, detected by antiserum no. 25 is demonstrated. As seen in Figs. 1 D and 2 A, this protein occurs in the absorbed antiserum no. 57 In most experiments, the α line will superimpose the *β_F-line as indicated in Fig 1 D*

In preliminary attempts to identify the α protein, reactions of complete identity with the main third antigen (MTA) described by *v Kleist & Burtin* (7) were observed. Further experiments, however exclude such identity Fig. 3 A gives the reactions when anti-MTA as well as MTA are included in our test system. Antiserum no. 25 reacts with the α protein in the absorbed antiserum no. 57 The reaction of apparently complete identity with MTA is due to α-protein present in the MTA preparation. The specific MTA reaction spurs over the α-protein line Excess of α-protein is demonstrated in the anti-MTA serum as well.

Immunoelectrophoresis also demonstrates that MTA and our *α-protein are different.* MTA moves slightly faster than the α-protein (Fig. 3 B)

Some findings in previous studies indicated that the α and β_F-proteins gave reactions of partial identity *in gel precipitation* experiments. The double-arched precipitin line in Fig 3 B also suggests that association exists. A series of crossed immunoelectrophoresis experiments was done to confirm these observations. PCA extracts *of colonic* carcinomas, or the β_F-fractions of this material, were run in two dimensions as shown in Figs. 4 and 5

In experiments shown in Fig 4 A, tumour

were freeze-dried and stored at −22 C until further tests were done

The β_I and β_E proteins found in the PCA extracts were separated on a Sephadex G 200 column eluted with 0 1 M phosphate buffer of pH 7 2–7.3 in 0 05 M NaCl Samples of 3–4 ml were collected and concentrated by freeze drying. The optical density of the eluted fractions was read at 254 nm. Protein concentrations were measured by the Folin method (9) Double immunodiffusion studies and immunoelectrophoresis were done in 1 per cent agarose gel If necessary weak precipitin lines were augmented by treatment with 4 per cent tannic acid as previously described (17) Crossed immunoelectrophoresis was run in 1 per cent agarose gel in the first dimension for 60 min at 8–10 volts/cm and in the second dimension for 20 h at 2 volts/cm on plates cooled to 15 C. In some of the experiments, an intermediate gel was interposed between the first and the second dimensional gel (1)

A barbital buffer of 0 075 M with 1 mM Ca lactate pH 8 6 was used

Antisera

Antiserum no 57 has been characterized in previous studies (16 17) The rabbit antiserum reacts with β_I and β_E proteins after absorption with PCA extract of normal liver and normal human serum It defines CEA determinants in the β_I protein and NCA determinants in both β_I and β_E In gel diffusion studies against these proteins, the two corresponding lines usually are separated the β_I line being closest to the antigen well

Antiserum no 25 was used in a previous study to detect β_E and the α protein This antiserum was produced in a rabbit immunized with eluate from the second peak of a Sephadex G 200 column run with PCA extract of colonic carcinoma. The unabsorbed antiserum shows strong activity against albumin and α-antitrypsin which was removed by absorption with 0 25 ml normal human serum per ml of antiserum. After absorption, the antiserum gives two precipitin lines in immunoelectrophoresis with PCA extracts of colonic carcinomas. The two lines characterize our α and β_E protein The activity of this antiserum will be further described in this paper

Antiserum no 90 was produced in a rabbit immunized with eluate from the first peak (CEA peak) of a Sephadex G 200 column run with PCA extract of colonic carcinoma After absorption with 0 25 ml normal human serum per ml of antiserum no precipitin line against normal human serum was seen In gel diffusion studies using PCA extract of colonic carcinomas, one precipitin line was seen in the β-region. This serum was used to characterize antigenic structure on CEA/NCA like materials.

Anti-MTA serum and MTA preparation were kindly supplied by c Kleist Anti-α-antitrypsin was produced in rabbits in our department. The other antisera including anti-α_1-antichymotrypsin, anti-α_1-acid glycoprotein, anti-α_2-antithrombin and antisera to a number of other proteins used in this study were commercially available (Behringwerke AG)

RESULTS

The β_E-protein was identified by antiserum no 57 (17) Figs. 1 and 2 show further reactivity of this protein with antisera nos. 90 and 25 These two antisera both give reactions of complete identity with the β_E-protein

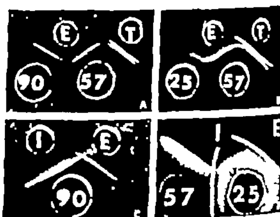


Fig 1 Detection of β_E-protein and characterization of antibody activity in antisera nos. 57 90 and 25 Double immunodiffusion in agarose gel.

Well 57 Antiserum no 57 absorbed with normal human serum and PCA extract of normal liver Activity against β_E or NCA in wells E and T and against β_I or CEA in wells I and T (the line closest to T) (17)

Well 90 Antiserum no. 90 absorbed with normal human serum. A, C Activity against NCA-determinants shared by β_I and β_E in wells I and E C Spurring shows β_E specific activity

Well 25 Antiserum no. 25 absorbed with normal human serum (D) and further absorbed with PCA extract of normal liver (B) B Activity against β_E reaction of complete identity with antiserum no 57 D No reaction with NCA-determinant on β_I in well I activity against the α protein present in the absorbed antiserum no 57 and activity against α and β_I protein in well E (superimposed lines)

Well E Pooled Sephadex G 200 fractions (No. 50 70 Fig 8) prepared from PCA extract of liver metastases of colonic carcinoma

Well I Sephadex G 200 fraction from peak one which contains β_I or CEA (17)

Well T PCA extract of colonic carcinoma.

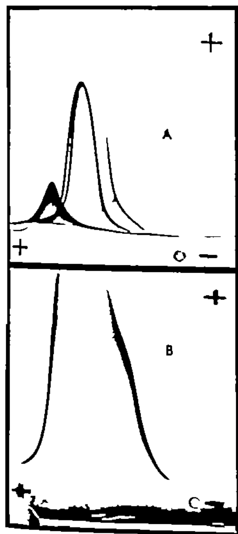


Fig 4 Demonstration of partial identity between the α- and β_E-proteins by crossed immunoelectrophoresis in agarose gel.

Material applied Pooled Sephadex G 200 fractions (Nos. 50–70, Fig. 8) prepared from PCA extract of liver metastases of colonic carcinoma, 2 mg prot./ml (A) and 4 mg prot./ml (B)

Antibodies Antiserum no. 25 unabsorbed and diluted 1/50 (A) and absorbed with normal human serum and diluted 1/50 (B)

First dimension run 8 V/cm for 60 min (A) and 10 V/cm for 60 min (B) Control by immunoelectrophoresis is shown at the bottom.

Second dimension run 2 V/cm overnight (20 h)

The two anodal peaks (A) is due to albumin and α-antitrypsin which are detected by the unabsorbed antiserum. The β_E-peak is weak and hardly visible in the first experiment (A) Experiment B demonstrates partial coprecipitation of the α- and β_E-proteins.

The α-protein and anti- α -antichymotrypsin also show identical electrophoretic mobility (Fig 7 B)

We did not observe any difference in the electrophoretic mobility of the α protein present in PCA extracts as compared with untreated serum The α-protein molecule did not either seem to undergo major size change due to PCA treatment, as treated as well as untreated preparations were eluted from the Sephadex G 200 column in the same region, presenting a peak in fraction no. 59 (Fig. 8) On the other hand, some charge heterogeneity was observed in electrophoretic experiments using this protein. The early Sephadex G 200 fractions showed a faster mobility and appeared more homogenous than the later ones (Fig. 6)

Also the β_E-protein showed electrophoretic heterogeneity the fast moving molecules being eluted in the early fractions (Fig 6) The elution pattern of α and β_E-proteins was quite similar β_E-protein being eluted slightly in front of the α-protein (Fig. 8)

Other proteins found in the same fractions were α_1-antitrypsin, the main third antigen (MTA) α-acid glycoprotein and α_2-antithrombin. There was no indication that β_E cross-reacts with MTA or any of these proteins. Carcino-embryonic antigen (CEA) or β_1 was eluted in the first peak (B) but not further tested in this study

DISCUSSION

A number of glycoproteins are found together with β_1 (CEA) and β (NCA) in the PCA extracts of malignant as well as normal tissue and serum. Immunological studies of these extracts show that apparently pure fractions often are contaminated with other glycoproteins of similar charge, molecular size



Fig 5 The reaction of anti MTA compared with the activity in antisera nos. 57 and 25. Double immunodiffusion and immunoelectrophoresis in agarose gel.

Well 57 Antiserum no 57 absorbed with normal human serum and PCA extract of normal liver

Well 25 Antiserum no. 25 absorbed with normal human serum.

Well E Pooled Sephadex G 200 fractions (Nos. 50–70 Fig 8) prepared from PCA extract of liver metastases of colonic carcinoma.

Well T PCA extract of colonic carcinoma

Well a MTA Anti-MTA serum

Well MTA MTA preparation

Anode to the left. The MTA line spurs over the line produced by the α-protein present in antiserum no. 57 and apparently also present in the MTA preparation as well as in the anti MTA serum. The MTA moves slightly faster than the α-protein in well E.

extract was tested against unabsorbed anti serum no 25 The two anodal peaks were identified as albumin and α_1 antitrypsin respectively The other peak is the α protein. The β_E-peak is partly seen as a trace reaction in this experiment.

In Fig 4 B the test conditions are changed The pooled β_E-fractions (E) of the tumour extract are used in higher concentration and antiserum no 25 has been absorbed with normal human serum to remove anti albumin and anti α_1-antitrypsin antibodies The distribution of α and β_E-proteins after the first run is shown at the bottom After the second run the two corresponding peaks are demonstrated. Deviation of the superimposed α and β_E-lines are seen on the right side of the peaks, indicating coprecipitation of the two proteins in this area.

The coprecipitation was mainly seen in the β-zone where the two proteins overlapped and some kind of interaction or formation of α/β_E-complexes in this zone could not be excluded Experiments were done to see whether coprecipitation or reaction of partial identity also could be demonstrated in other zones. In Fig 5 fast moving α protein was run into the antibody-containing gel together with slow-moving β_E-protein A reaction of partial identity is still seen in the zone where the two proteins move together The spur formation demonstrates specific α protein determinants, while the weak precipitin arch intersecting the cathodal peak in Fig 5 A is interpreted as due to specific β_E-protein determinants which also are seen in Fig. 4 B.

In Fig 5 B an intermediate gel containing α protein is applied between the gel strip from the first run and the antibody-containing gel in order to identify the α line.

A number of PCA extracts of normal as well as tumour material have been tested. If tested in low concentration by immunoelectrophoresis, reaction of identity will be marked while the specific α and β_E-determinants may be difficult to demonstrate. In higher concentration, reaction with α and β_E-specific determinants dominates and covers the reaction of partial identity Some variations among preparations of different tumours were also observed, e.g Fig 6, where E shows no spurring over the α-line compared with E_2 which has a marked spur

Identification and characterization of the α protein was done in a series of experiments which included specific antisera to more than 30 different serum proteins.

Antiserum no 25 and anti α-antichymotrypsin give lines of complete identity when tested against the α protein (Fig 7 A)

The heterogeneity observed in gel filtration as well as in immunoelectrophoresis shows that the β_E-protein in the PCA extract behaves rather as a mixture of closely related molecules than as a homogeneous population of molecules, being slightly different in electric charge and molecular weight. The larger molecules, eluted early from the Sephadex G 200 column, were found to have a slightly more negative charge than the smaller ones. Gel filtration experiments would suggest an average molecular weight close to α_1-antichymotrypsin, i.e. in the range of 60–80,000 daltons. These phenomena, however were found in PCA extracts which may have altered the structure of the β_E-protein. The concentration of β_E-proteins in untreated material was too low to be studied by our methods.

Antiserum no. 25 reacts with β_E-specific determinants, but not with NCA which were detected by antiserum no. 57 both on β and β_E. Otherwise, these two antisera give reactions of complete identity and react with β_E-molecules of similar electrophoretic mobility. Therefore, it seems unlikely that the two antisera should detect different substances or different subpopulations of β_E-molecules.

The α protein was identified as α antichymotrypsin or a closely related substance. The criteria for this identification were reaction of complete identity with specific anti-

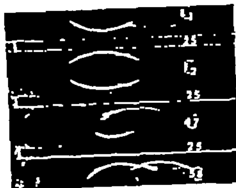


Fig. 6. Demonstration of heterogeneity in different preparations and fractions of the α- and β_E-proteins by immunoelectrophoresis in agarose gel.

Trough 25. Antiserum no. 25 absorbed with normal human serum.

Well E1. Pooled Sephadex G 200 fractions (Nos. 50–70, Fig. 8) prepared from PCA extract of liver metastases of colonic carcinoma (T 33).

Well E2. Material prepared in the same way from another liver metastases of colonic carcinoma (T 32).

Well 47, 58. Sephadex G 200 fractions nos. 47 and 58 (Fig. 8) prepared from PCA extract of liver metastases of colonic carcinoma (T 32).

Anode to the left. Differences in the electrophoretic pattern of E1 and E2 preparations are seen. The early β_E-fractions migrate faster than the later one. The early fraction of the α-protein is of a more homogeneous appearance and moves slightly faster than the later one.



Fig. 7. Identification of the α-protein by double immunodiffusion and electrophoresis in agarose gel.

Well αch. Anti-α-antichymotrypsin (Behringwerke).

Well 25. Antiserum no. 25, absorbed with normal human serum.

Well 47, 58. Sephadex G 200 fractions nos. 47 and 58 (Fig. 8) prepared from PCA extract of liver metastases of colonic carcinoma (T 32).

Well 57. Antiserum no. 57 absorbed with excess of α-protein (see Fig. 2).

Anode to the left. Antiserum no. 25 and anti-α-antichymotrypsin give reactions of complete identity with the α-protein present in antiserum no. 57 as well as with fractions nos. 47 and 58. The electrophoretic mobility is also identical.

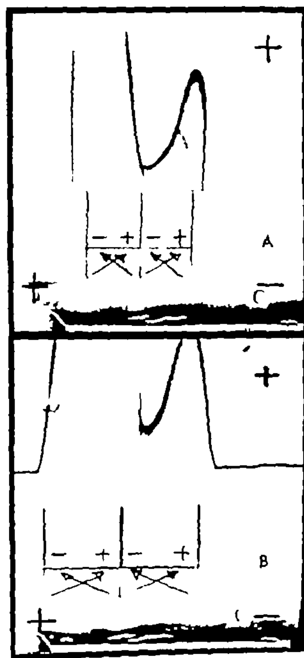


Fig 5 Demonstration of partial identity between the α- and β_E-proteins by crossed immunoelectrophoresis in agarose gel Comparison of slow β- and fast α zones.

Material applied Pooled Sephadex G 200 fractions (Nos. 50–70 Fig. 8) prepared from PCA extract of liver metastases of colonic carcinoma, 2 mg prot./ml (A) and 4 mg prot./ml (B)

Antibodies Antiserum no 25 absorbed with normal human serum and diluted 1/50.

Intermediate gel In experiment B, a gel strip with the α-protein was applied between the antigen- and antibody-containing gels in order to identify the α precipitin line (Antiserum no. 37 diluted 1/100 with excess of α-protein but no β_E-protein, was used for this purpose The anti-β_E activity was diluted too much to give precipitin lines)

First dimension run 8 V/cm for 60 min (A) and 10 V/cm for 60 min (B) Control by immunoelectrophoresis is shown at the bottom The gel strips were cut and turned around as indicated (arrows) to examine the interaction of the fast α- and slow β_E-proteins. Before the second run, the proteins in the two sections were allowed to diffuse together for 105 min (A) and 90 min (B)

Second dimension run 2 V/cm overnight (20 h) Reactions of partial identity are noticed where the two peaks intersect. In experiment (A) also an β_E arch is seen within the cathodal α-peak.

or structure Such impurities may easily confuse the results of immunological experiments

Furthermore, cross-reactions between substances like β_1 and β_E or others may cause problems of identification It is therefore important to detect such cross-reactions which may give false results in simple immunological tests like double immunodiffusion experiments where precipitin lines often will superimpose each other Demonstration of cross reaction could also indicate that two substances had some functional or biological relationship

One of the best methods to detect cross-reaction seem to be crossed immunoelectrophoresis or modifications of this method (1) Antisera are needed which possess activity against specific determinants as well as determinants shared by the two substances. Antisera which were weakly or partly absorbed in order to preserve such activities were therefore used in our experiments.

The β_E-protein was defined by antiserum no. 57 and found to cross-react with CEA (16 17) It shows electrophoretic heterogeneity in the β–α zone and seems to be identical with a glycoprotein or group of glycoproteins which have been described as NCA (6) NGP (10) CEX (4) CCEA-2 (14) and CCA III (12)

Sedimentation constants of 3–4 S have been found (6) and a molecular weight of 60 000 daltons reported (3 5) Function and biological activity is not known.

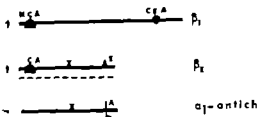


Fig. 9 A model of the demonstrated antigenic relationship between β_I, β_{II} and α-antichymotrypsin
——— Molecules of β_I, β_{II} and α-antichymotrypsin with the associated determinants. Possible β_{II}-variants (subpopulations)

α_1-antichymotrypsin (Behringwerke) similar electrophoretic mobility and similar elution pattern in gel filtration experiments. Chemical analyses and analyses of biological activity are needed for a complete identification and classification as α_1-antichymotrypsin which acts as an inhibitor of chymotrypsin or chymotrypsinlike substances (2)

The previously observed cross-reaction between β_{II}- and α proteins was confirmed by crossed immunoelectrophoresis. Coprecipitation of the two proteins was found and could be demonstrated in different electrophoretic zones. The finding may indicate that the two substances have some determinants or structure in common though some kind of interaction e.g complex formation between the two proteins cannot be excluded.

Complex-formation between protease and proteaseinhibitors is known to occur (2) Such complexes could possibly explain the observed cross-reaction between α-and β_{II}-molecules. The fact that the α-protein was found in the absorbed antiserum no 57 together with anti-β (NCA) antibodies, however would exclude the presence of complexes of α-protein and intact β_{II}-molecules. Such complexes, containing active NCA-determinants, would have neutralized the anti-β activity On the other hand, the presence of complexes consisting of α-protein and β_{II}-fragments or variants of β_{II} which lack NCA determinants, cannot be excluded.

The association of β_{II} with α antichymotrypsin raises the question whether these two substances are functionally related or part of an enzyme/enzyme-inhibitor system. Data have been presented that CEA, which is antigenically related to β_{II} has carboxylesterase activity (11) though recent observation shows that this question may be controversial (13) Some preliminary experiments were instituted with a view to learning whether chymotrypsin activity could be detected in the β_{II}-preparation. Chymotrypsin substrates, like benzoyl-phenylalanine-β naphthyl ester and acetyl-phenylalanine-β naphthyl ester were used in an immuno-enzyme assay (15) Negative reactions were found in these experiments which will not be described in detail. They were done only in PCA extracted fractions and a possible enzyme activity may have been destroyed. Further studies of β_{II}-protein are needed to evaluate this question.

Our experiments confirmed the observations that β_{II}-like proteins also possess their own specific determinants in addition to the common antigenic structure shared with CEA-molecules (6) Such distinct determinants were demonstrated by antiserum no. 90 as well as no. 25 Also the α-protein possessed antigens which were specific for this protein in addition to the determinants shared with the β_{II}-protein. A model of the antigenic relationship demonstrated in these substances, compared with CEA, is shown in Fig 9 The determinants shared by the β_{II}- and α-protein are different from the NCA determinants which are not detected by antiserum no. 25

The author wishes to thank Dr *S* [illegible] *Kleist* Institut de Recherches Scientifiques sur le Cancer Villejuif for providing the MTA preparation and anti-MTA serum. Thanks are given to Mrs. *I ger Lill S andsen* for skilful technical assistance.

REFERENCES

1 *Axelsen N H., Krøll, J & Weeke B* (Eds): A manual of quantitative immunoelectrophoresis Methods and applications. Scand. J Immunology 2 47 99 suppl. 1 1973

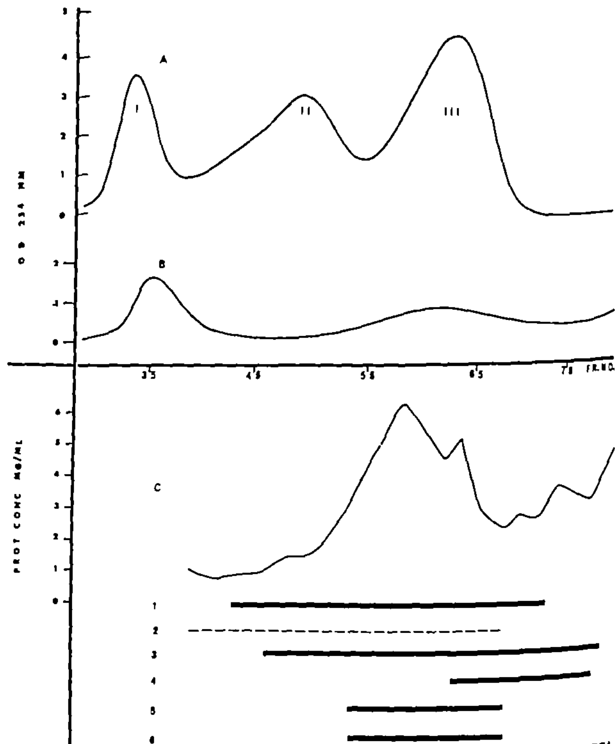


Fig 8 Substances detected by immunodiffusion in the Sephadex G 200 fractions prepared from PCA extract of liver metastases of colonic carcinoma

Upper curve Reference run of a normal human serum pool.

Middle curve The PCA extract of tumour peak B contains β_1 (CEA) (not tested)

Lower curve Protein concentration measured by the Folin method in the concentrated fractions.

1 α_1-antichymotrypsin.
2 β_E-protein
3 α_1 antitrypsin.
4 Main third antigen (MTA) (7 8)
5 α-acid glycoprotein.
6 α_2-antithrombin (antithrombin III)

Acta path. microbiol. scand. Sect. C, 84 245–249 1976

STUDIES ON THE *TREPONEMA PALLIDUM* IMMOBILIZING ACTIVITY IN NORMAL HUMAN SERUM

5 *On the Protective Role against Syphilis*

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Hederstedt, B Studies on the *Treponema pallidum* immobilizing activity in normal human serum. 5. On the protective role against syphilis. Acta path. microbiol. scand. Sect. C, 84 245–249 1976.

It was shown that *T pallidum* immobilized *in vitro* by normal serum had lost its infectivity in rabbits. This finding suggested that the immobilizing activity of normal serum might play a role in the natural resistance of man against infections with *T pallidum*. However the results of serological studies of acquired early syphilis in man and of experimental syphilis in cynomolgus monkeys did not present evidence that the immobilizing activity of normal serum protected against syphilis infection.

Key words *Treponema pallidum*; immobilizing activity normal human serum; protective role syphilis.

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A method for quantitative determination of the *T pallidum* immobilizing activity of human normal serum (NS) has been developed (*Hederstedt* 1974) IgM antibody complement and lysozyme were found to take part in the immobilization reaction (*Hederstedt* 1976 a) The determination of the activity of the naturally occurring IgM immobilizins was not influenced by coexisting immune serum (IS) antibodies of the IgG class and *vice versa* Finally the *T pallidum* immobilizing activity demonstrated in normal sera from human individuals and cynomolgus monkeys was found to be of about the same frequency 75–85 per cent (*Hederstedt* 1976 c)

The present work analysed the role of the immobilizing activity in human and monkey NS for the protection against infection by *T pallidum*.

MATERIAL AND METHODS

Sera from man and *cynomolgus* monkey were obtained and stored as previously described (*Hederstedt* 1976 c)

The T pallidum *immobilizing activities of normal sera (NS) and of immune sera (IS)* were determined as previously described (*Hederstedt* 1974 *Hederstedt & Skog* 1964) The serum titres were expressed as the reciprocal of the final serum dilutions that gave 50 per cent immobilization and were referred to as the NS and the IS immobilizing titre, respectively All the various samples from the same person or animal were titrated at the same time.

The infectivity of treponemes immobilized in vitro by human normal serum. Equal amounts of

2 *Bender M L. & Killheffer J V* Chymotrypsins CRC Critical Reviews in Biochem. *1* 149–199 1973

Burtin P Chavanel G & Hirsh Marie H Characterization of a second normal antigen that cross-reacts with CEA. J Immunol. *111* 1926–1928 1973

Darcy D A Tubervville C & James R. Immunological study of carcinoembryonic antigen (CEA) and related glycoproteins. Br J Cancer *28* 147–160 1973

Fuks A Banjo C., Shuster J Freedman S O & Gold P Carcinoembryonic antigen (CEA) Molecular biology and clinical significance. Biochimica et Biophysica Acta *417* 123–152 1974

Kleist S von Chavanel G & Burtin P Identification of an antigen from normal human tissue that cross-reacts with the carcinoembryonic antigen. Proc. Nat. Acad. Sci. U.S.A *69* 2492–2494 1972.

Kleist S von & Burtin P Isolation of a fetal antigen from human colonic tumours. Cancer Res. *29* 1961–1964 1969

Kleist S von King M & Burtin P Isolation and characterization of a third main antigen of human colonic tumours. Joint Meet. Europ Soc. Immunol. Strasbourg, 1973 p 40 (Abstract)

Lowry O H Rosebrough N J Farr A L. & Randall R J Protein measurement with the folin phenol reagent. J biol. Chem. *193* 265–275 1951

Mach J P & Pusztaszeri G Carcinoembryonic antigen (CEA) Demonstration of a partial identity between CEA and normal glycoprotein. Immunochemistry *9* 1031 1034, 1972

11 *Munjal, D Zamcheck N Kupchik H Z & Saravis C A* Correlation of carcinoembryonic antigen content with carboxylesterase activity in benign and malignant human tissue Cancer Res. *34* 2936–2939 1974

12 *Newman E. S., Petras S E., Georgiade, A & Hansen H J* Interrelationship of carcinoembryonic antigen and colon carcinoma antigen III Cancer Res. *34* 2125–2130, 1974

13 *Thomas P & Westwood J H* Carboxyl esterase activity of carcinoembryonic antigen Br J Cancer *32* 401–402, 1975.

14 *Tuberville C Darcy D A Laurence D J R., Johns E. W & Neville M* Studies on carcinoembryonic antigen (CEA) and a related glycoprotein CCEA 2. Preparation and chemical characterization. Immunochemistry *10* 841–843 1973

15 *Uriel J* Characterization of enzymes in specific immunoprecipitates. Ann. N Y Acad Sci. *103* 956–979 1963

16 *Ørjasæter H Fredriksen G & Liavåg I* Studies on carcinoembryonic and related antigens in malignant tumours of colon-rectum Acta path. microbiol scand Sect. B, *80* 599–608 1972

17 *Ørjasæter H* Demonstration of carcinoembryonic antigens (CEA) non-specific cross reacting antigens (NCA) and an associated alpha protein in normal human tissues and fluids by Immunodiffusion techniques. Acta path. microbiol. scand, Sect B, *82* 387–395, 1974

TABLE 2. *The Susceptibility of Cynomolgus Monkey to Infection with 9.0 × 10[1] T. pallidum Organisms in Relation to their NS Immobilizing Activity*

NS immob. titre prior to inoculation range	NS immob. titre prior to inoculation mean	Monkey number	Time in weeks from infection to demonstration of IS antibodies	Time in weeks from infection to demonstration of NS antibodies
		1	16	7
		2	– (†6)	–**
		3	28	15
	<2.0	4	15	16
		5	–	7
		6	–	8
		7	– (†20)	
		8	19	
		9	– (†17)	
4.0–16.5	11.1	10	– (†15)	
		11	–	
		12	15	

† = the animal died
** = no antibodies were detected.

animals, as reflected by the period of time between inoculation and the appearance of skin reactions or IS (IgG) antibodies. The animals initially lacking NS immobilizing activity developed such NS activity within four weeks following inoculation.

Seventeen weeks after inoculation, the NS titres of animals in both groups were of about the same magnitude and so were also their IS titres.

Control experiments revealed that *T. pallidum* immobilizing activity of the NS type could regularly be demonstrated within one week following inoculation of normal rabbit testicle tissues.

The same type of experiment as that described above was repeated with monkeys which received small infective doses of 9.0 × 10[1] treponemes. None of the animals showed skin reactions after inoculation of this small dosage. As appears from Table 2, IS antibodies developed in five animals, namely in three of the animals initially lacking NS immobilizing activity and in two of the animals possessing NS immobilizing activity—after 15–28 weeks.

This interval of time was more than twice as long as that to elapse after inoculation into animals of 9.0 × 10[7] treponemes. In the group of monkeys initially lacking NS immobilizing activity such NS activity developed 7–16 weeks after inoculation. Four animals died at the time of sampling of blood i.e. 6–20 weeks after inoculation (Table 2).

In control animals inoculated with a diluted suspension of normal rabbit testicular tissues, no NS immobilizing activity was demonstrated during the observation period.

T. pallidum *immobilizing activity of the NS type in human individuals who according to reports had contact with patients with early syphilis.* The NS immobilizing titre of sera from eight patients who developed signs of syphilis 7–45 days (mean 25 days) after the serum samples had been taken did not differ from the NS titre of sera from 14 individuals who did not reveal syphilis symptoms within six months after the first serum samples had been taken (Table 3).

T. pallidum *immobilizing activity of the NS and IS type in human individuals re-infected with syphilis.* Sera from ten patients with early syphilis who 4–24 months (mean 12 months) after treatment appeared to have been re-infected were tested for immobilizing activity. The mean NS titre 4–25 weeks

TABLE 3. *The NS Immobilizing Activity in Sera of Human Syphilis Contact*

Exposure followed by	Number of sera	NS immob. titre range	NS immob. titre mean
Symptoms	8	2.4–9.0	4.4
No symptoms	14	2.0–16.4	4.4

TABLE 4. *The Titre of the NS Immobilizing Activity and the TPI Titre of Human Individuals Re-infected with Syphilis*

Number of sera	NS immob. titre range	NS immob. titre mean	TPI titre range	TPI titre mean
10	2.6–11.8	6.0	5–26	8.8

treponemal suspension containing 9.0×10^{6} living treponemes per ml and unheated or heated human NS were incubated at 35 C for two hours. Following this incubation, the unheated NS was found to immobilize all the treponemes while heated NS would leave all the treponemes motile. The samples were inoculated intracutaneously in a shaved area on the backs of six rabbits. In one experiment the rabbits received three injections at different sites, using 0 1 ml of each sample. The animals were observed daily for the development of skin reactions. In a second experiment, the samples with motile and immobilized treponemes, respectively were injected into different rabbits. Blood was drawn monthly from the animals and the serum samples were tested for IS antibodies. The rabbits were sacrificed after six months and the popliteal lymph nodes of each animal were homogenized in 1 0 ml of saline. Each homogenate was inoculated into the testis of a new rabbit. Blood was drawn from these rabbits every second month for six months and the sera were tested for IS antibodies.

The susceptibility of the cynomolgus monkey to infection with T. *pallidum was studied in monkeys* lacking immobilizing NS activity and in monkeys possessing immobilizing NS activity.

In one experiment each animal was inoculated *intracutaneously at four sites of a shaved area* on their backs with 0 25 ml (at each site) of a suspension containing 9.0×10^{7} motile treponemes per ml. Blood was drawn 4 weeks after inoculation and thereafter weekly for 12 weeks. The sites of inoculation were inspected for skin reactions twice weekly.

Control animals lacking NS immobilizing activity were inoculated with a suspension of normal rabbit testicular tissues, prepared in the same way as the above treponemal suspension. Blood was drawn weekly during five weeks.

In a second study monkeys were inoculated as described above but 9.0×10 treponemes were given.

The control animals, lacking NS immobilizing activity were inoculated with a suspension of normal rabbit testicular tissues diluted 1 10 000 in the same medium as that used for the treponemes.

Blood was drawn weekly after inoculation during 23 weeks and the animals were finally bled to death 28 weeks after inoculation.

RESULTS

In the six rabbits inoculated in the skin both with motile treponemes and with treponemes immobilized by human NS erythemas or ulcerations appeared at all those sites where motile treponemes were injected within 11 18 days after inoculation. No skin reactions were observed at sites where immobilized treponemes had been given.

In another experiment, three rabbits were given immobilized treponemes while three rabbits received motile treponemes. Within *two months after the inoculation* IS (TPI) antibodies were demonstrated in the three animals injected with the motile treponemes, while no IS antibodies were detected in the other animals even after six months. When homogenates of lymph nodes from the six rabbits were inoculated into six other rabbits, IS antibodies were demonstrable within two months in animals inoculated with lymph nodes from the IS positive rabbits, while no serum antibodies were demonstrated in the other animals during the observation period.

The susceptibility of cynomolgus monkeys to infection with various numbers of T. pallidum. Monkeys lacking NS immobilizing activity and monkeys possessing NS immobilizing activity were *inoculated with suspensions* of various numbers of *T. pallidum*. The results of the experiment in which a large inoculum (9.0×10^{7} treponemes) was used (Table 1) showed that the susceptibility to infection did not differ in the two groups of

TABLE 1 *The Susceptibility of Cynomolgus Monkeys to Infection with* 9.0×10 T. *pallidum Organisms in Relation to their NS Immobilizing Activity*

NS immob titre prior to inoculation		Number of monkeys	Time in week from infection to the demonstration of:				
			Skin reaction		IS antibodies		NS antibodies
range	mean		range	mean	range	mean	
	<2.0	6	4–7	5 2	6 10	8.1	<4
6.0–11.3	8 1	6	4 7	5 0	6–10	8.8	–

little or no protective power in monkeys against infections with *T. pallidum*.

The studies in monkeys as well as in man (*Hederstedt* 1976 c) revealed that the titres of the natural immobilisins increased during the syphilis infection. This finding suggests that antigens of the rabbit treponemal strain as well as the "wild" human strains can initiate the formation of natural immobilisins or/and that the host tissue reaction caused by the treponemes liberate cross-reacting antigen capable of producing immobilisins.

In the present study the NS immobilizing titres of individuals who according to reports had contact with patients with early syphilis and who contracted the disease were not found to be lower than those in contacts who never showed signs of the disease. Though based on a small series, 22 cases, this finding might also argue against the protective effect of the immobilizing activity of NS.

The same conclusion might be drawn from the results obtained by observation of some patients with early syphilis who were followed serologically after treatment and who during this observation period appeared to have been re-infected. On the basis of available epidemiological and serological data it is reasonable to suggest that some of these individuals, at the time of re-infection, had NS immobilizing activity of titres higher than the mean NS titre of healthy human individuals, as well as demonstrable amounts of IS (TPI) antibodies.

The role of humoral immunity in cases of syphilis has long been unclear. Recent studies of the effect of passive immunization on experimental syphilis in the rabbit (*Sepetjian* et al. 1973. *Turner et al.* 1973) revealed, however, that the IS antibodies might have a relative protective effect. In the experiments by *Sepetjian et al.* (1973) the infected rabbit developed an asymptomatic infection, while *Turner et al.* (1973) found in their rabbits a "prolongation of incubation, attenuation of chancres with accelerated healing and a decreased incidence of metastatic lesions".

According to the present study there is reason to believe that the NS immobilizing activity does not affect the incidence of acquired syphilis in man.

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REFERENCES

1. *Hederstedt, B. & Skog, E.* Quantitative *Treponema pallidum* immobilization (TPI) test in early syphilis. Acta derm.-venerol. *44*: 82–90, 1964.
2. *Hederstedt, B.* Studies on the *Treponema pallidum* immobilizing activity in normal human serum. 1. A method. Acta path. microbiol. scand. Sect. B, *82*: 185–200, 1974.
3. *Hederstedt, B.*, Studies on the *Treponema pallidum* immobilizing activity in normal human serum. 2. Serum factors participating in the normal serum immobilization reaction. Acta path. microbiol. scand. Sect. C, *84*: 135–141, 1976 a.
4. *Hederstedt, B.*, Studies on the *Treponema pallidum* immobilizing activity in normal human serum. 3. A comparative study on the kinetics of the immobilization reaction by normal and immune sera. Acta path. microbiol. scand. Sect. C, *84*: 142–147, 1976 b.
5. *Hederstedt, B.* Studies on the *Treponema pallidum* immobilizing activity in normal human serum. 4. On the importance for the outcome of the conventional TPI test. Acta path. microbiol. scand. Sect. C, *84*: 148–152, 1976 c.
6. *Magnuson, H. J., Thomas, E. W., Olansky, S., Kaplan, B. I., De Mello, L. & Cutler, J. D.* Inoculation syphilis in human volunteers. Medicine *35*: 33–82, 1956.
7. *Putkonen, T.* Risk of infection, incubation period, and first clinical signs in syphilis. Acta derm.-venerol. *31*: 605–614, 1951.
8. *Sepetjian, M., Salussola, D. & Thivolet, J.* Attempt to protect rabbits against syphilis by passive immunization. Brit. J. vener. Dis. *49*: 335–337, 1973.
9. *Turner, T. B., Hardy, J. P. H., Newman, B. & Nell, E.* Effects of passive immunization on experimental syphilis in the rabbit. WHO/VDT/RES *73*: 308, 1973.
10. *van Wernsdorff, A. J.*: The incidence of infection in contacts of early syphilis. J. vener. Dis. Inform. *29*: 132–137, 1948.

(mean 12 weeks) before *symptoms of re-infection* appeared was found to be 6.0 (Table 4) which was higher than the titre in healthy individuals (Table 3). Moreover in each of these sera *low titres of immobilizing* antibodies of the IS type were demonstrated.

DISCUSSION

Some observations suggest that natural resistance against syphilis may develop in man. *One example of this is* apparent from the results obtained by *Werssouets* (1948) and *Putkonen* (1951) in studies of patients who had contact with early syphilis. These authors *found that less than 50 per cent of human* individuals exposed to syphilis appeared to contract the disease. On the other hand some reports on experimental syphilis in man indicate *that the non-syphilitic individual is* highly susceptible to infection with *T. pallidum* if the treponemes are introduced below the skin. Thus, intracutaneous inoculations of "*approximately 57 organisms*" *applied to* eight non-syphilitic human volunteers in the Sing Sing Prison, New York, produced infections in 50 per cent of the volunteers (*Magnusson et al.* 1956).

The main purpose of the present study was to determine whether the activity of human NS which immobilized the treponemes *in vitro* even more rapidly than human IS (*Hederstedt* 1976 b) might play a role in the natural resistance of man against infections with *T. pallidum*.

The finding in rabbits (the animal species usually used for infectivity tests in studies of syphilis) that treponemes, immobilized *in vitro* by unheated human NS had lost their infectivity indicates that this activity of NS might be of importance for the protection against syphilis infection.

In a previous study (*Hederstedt* 1976 c) it was found that NS immobilizing activity in the cynomolgus monkey occurred at about the same frequency and titre as in man. In addition, the period of time between exposure to *T. pallidum* and the first appearance *of skin reactions* as well as IS immobilizins was of approximately the same length in the cynomolgus monkey and in man (*Hederstedt & Skog* 1964). The monkey was therefore *suggested to be suitable for a study under* controlled conditions, of the possible protective role of the NS immobilizing activity.

Using a heavy inoculum of *T. pallidum*, skin reactions and TPI antibodies would develop at approximately the same time whether the monkeys initially lacked or possessed natural serum immobilizins. This finding however *did not prove that the natural* immobilizins were non-protective. In fact, the heavy treponemal inoculum used, contained also high concentrations of rabbit testicular tissues. *When inoculated separately the* rabbit tissues evoked the development of immobilizins of the NS type within one week, indicating that an antigen in the rabbit tissues *was immunologically similar or identical* to the antigen of *T. pallidum* reacting with immobilizins of NS (*Hederstedt*, to be published).

To minimize the immobilizin production by coexisting rabbit testicular tissue and to make the test more sensitive as regards the detection of a possible protective capacity of the naturally occurring serum immobilizins, a more diluted treponemal suspension was used. This procedure did not reveal any differences in the development of symptoms or IS antibodies among the animals whether or not they initially had NS immobilizins.

Though the number of animals was small, some conclusions can be drawn from the results of the present experiments. Using a large inoculum (9.0×10^7) of treponemes, the NS immobilizing activity of relatively high titre could not prevent the treponemes from infecting the animals. An inoculum of 9.0×10^3 treponemes was too small *to produce* skin reactions in the monkey which initially possessed or lacked NS immobilizins and TPI antibodies developed in *only some* of the animals. These *observations* might imply that this low dose was close to the 50 per cent infectious dose for monkeys, in other words, the natural immobilizins had

centrifugation procedure, the treponemes were killed. The suspension contained 6.5×10^7 treponemes/ml the dry weight was 0.06 mg per ml.

The animal tissue homogenates were prepared from normal rabbit testicles and liver as well as from testicles of guinea pigs and white rats, as described by *Gajdusek* (1958) The dry weight of the various tissue preparations varied between 20 and 33 mg per ml. The homogenates were stored in small aliquots at −60° C.

The HeLa cells were cultivated in Eagle's medium + 20 per cent calf serum for two to three days The cells were trypsinized and washed three times in aliquots of isotonic saline solution. The dry weight of the final suspension was 40 6 mg per ml The cells were used either intact or as a sonicate

The T pallidum *immobilizing activities of the normal and immune sera as well as the haemolytic complement activity of the sera* were determined as described previously (*Hederstedt* 1961 *Hederstedt & Skog* 1964 *Hederstedt* 1974)

The absorption of the immune serum with T pallidum was performed in the same way as absorption of normal sera (*Hederstedt* 1976) i e. two parts of the *T pallidum* suspension, partially purified by density gradient centrifugation, and seven parts of the serum were mixed. The absorption procedure was carried out at 0 C or 35 C.

The absorption of normal sera with tissue homogenates was carried out at 0 C for 30 min. Equal volumes of normal serum (NS) and sorbent were used If not otherwise specified, the absorbed serum was tested for immobilizing activity after centrifugation at 20,000 *g* for 60 minutes at 0 C. The sorbent was considered to have antibody combining properties if the immobilizing activity of the absorbed NS was significantly reduced Simultaneously residual haemolytic complement activity should be demonstrable in the reaction mixture.

The inoculation of tissue homogenates HeLa cells and treponemes into cynomolgus monkeys 2 ml of the various preparations were applied subcutaneously by a single dose into the back of the animal. Blood was drawn from the animals before inoculation and one and two weeks after inoculation. The inoculum was considered to be antigenic for monkeys if the animals developed immobilizing activity of the NS type within two weeks.

RESULTS

The Relationship between the Antigens Associated with the Immobilizing Antibodies of Normal Serum (NS) and Immune Serum (IS)

Unheated human syphilis sera (IS) were absorbed at 0 C for 30 minutes with a suspension of purified and killed *T pallidum* (by centrifugation through a density gradient) Prior to the absorption, the IS possessed immobilizing activity of the NS as well as of the IS type, the titres of the two activities being of approximately the same magnitude.

The absorption deprived the IS of their immobilizing activity of the NS type, leaving their activity of the IS type as well as their total haemolytic complement activity unaffected.

The absorbed IS were subjected to repeated absorption with treponemes at 0 C or alternatively at 35 C for an additional 20 hours. These prolonged absorption procedures did not significantly reduce the titres of the immobilizing activity of the IS type.

The effect of these absorptions on the immobilizing titres of one out of three representative IS is seen in Table 1

TABLE 1 *The Effect of Absorption with* T pallidum *on the Immobilizing Titres of the NS and IS Type Respectively of Human Syphilis Serum*

	Immobilizing titre (IT_{50}) of NS type	Immobilizing titre (IT_{50}) of IS type	Complement haemolytic titre (CH_{50})
Unabsorbed	15 2	13.5	212
Absorbed 0° C, 30 min	<2 6	15.7	200
Absorbed 35 C, 30 min	<2.6	15.2	157
Absorbed 0° C, 30 min + absorbed 0° C, 20 hours		11 7	182
Absorbed 0° C, 30 min + absorbed 35 C, 20 hours	–	14.8	108

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STUDIES ON THE *TREPONEMA PALLIDUM* IMMOBILIZING ACTIVITY IN NORMAL HUMAN SERUM

6 *Antigenic Relationship between* T pallidum *and Various Mammalian Tissues*

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Hederstedt B Studies on the *Treponema pallidum* immobilizing activity in normal human serum. 6 Antigenic relationship between *T pallidum* and various mammalian tissues. Acta path. microbiol. scand Sect. C 84 250–254 1976

The immobilizing IgM antibody of normal serum and the IgG antibody of syphilis serum reacted with different antigens of *T pallidum* The normal serum antibody to *T pallidum* cross-reacted with antigens of various mammalian tissues.

Key words *Treponema pallidum* immobilizing activity normal human serum, antigenic relationship

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Following syphilis infection in man *T pallidum* immobilizing serum antibodies of the IgG variety are detected only in the TPI test (*Laurell & Hederstedt* 1958 *Julian et al* 1969)

In a modified TPI test (*Hederstedt* 1974) the majority of human normal sera (NS) was found to possess *T pallidum* immobilizing activity achieved by the joint action of IgM antibodies and complement (*Hederstedt* 1976 a)

The determination of the titre of the IgG immobilisins in human syphilis serum (IS) was not influenced by coexisting naturally occurring immobilizing IgM antibodies. To all appearances, determination of the immobilizing activity of the NS type in IS was not either influenced by IgG immobilisins (*Hederstedt* 1976 b)

It was found that the two types of antibodies were reacting with different antigens and that immobilizing activity of the NS type developed in monkeys upon inoculation of killed *T pallidum* as well as various mammalian tissues.

MATERIALS AND METHODS

The human normal sera (NS) and *Immune sera (IS)* were obtained and stored as previously described (*Hederstedt* 1976 b)

The monkey sera were obtained from cynomolgus monkeys by heart puncture The sera were stored in the same manner as the human sera.

The cynmolgus monkeys used in the inoculation experiments weighed 1300–2300 g. The monkeys selected were exclusively those in which unheated sera did not exhibit an immobilizing activity on *T pallidum*

The suspension of T pallidum *purified by density gradient centrifugation* was prepared as described before (*Hederstedt* 1976 a) Following the

TABLE 3 *The Effect on the* T. pallidum *Immobilizing Activity of Four Human NS (I–IV) after Absorption with* T. pallidum *and various Tissues*

	NS immobilizing titre (IT_{50})				Reduction (in per cent) of complement haemolytic activity of serum
	I	II	III	IV	I–IV mean values
Unabsorbed	10.1	14.1	16.9	18.2	
Absorbed with					
Killed *T. pallidum*	<4.0	<4.0	<4.0	<4.0	9
HeLa cells	11.2	16.0	17.2	16.9	42
Monkey testicle	11.2	16.0	16.0	22.4	14
Rabbit testicle	<4.0	<4.0	<4.0	18.5	11
Rabbit liver	<4.0	5.6	5.2	16.9	14
Guinea pig testicle	7.6	4.5	7.6	<4.0	50
White rat testicle	<4.0	<4.0	<4.0	7.5	10

treponemal concentration of such suspensions might be responsible for this failure.

It is known from an earlier study (*Hederstedt* 1976 a), however, that suspensions of treponemes that had been concentrated and purified by centrifugation through a density gradient were able to absorb the *T. pallidum* immobilizing activity out of NS. In the present study, such absorptions did not reduce the *T. pallidum* immobilizing IgG antibody titre of syphilis serum. Accordingly, it was suggested that the gradient centrifugation procedure might destroy or remove the treponemal antigenic determinants of the immobilizing IgG antibodies of syphilis serum. If so, it is consistent with the finding by *Metzger et al.* (1969) that this antigen is a protein that is very sensitive to various chemical and physical treatments.

In a study of experimental syphilis in monkeys (*Hederstedt* 1976 c), it was found that cynomolgus monkeys, lacking NS immobilizins and inoculated with a single dose of killed *T. pallidum* or normal rabbit testicular homogenate, developed immobilizing activity of the NS type within one week.

In the present study it was found that the inoculation of tissues other than testicles and of testicular tissues from animal species other than *rabbit* also evoked the production of *T. pallidum* immobilizing antibodies in cynomolgus monkeys. These results suggested that a so-called ubiquitous antigen might be responsible for these antibodies.

The results of absorptions of human normal sera with *T. pallidum* or tissues from various animals were in most respects consistent with those obtained in the inoculation experiments. The immobilizing activity in one human NS, however, was completely abolished by the absorption with *T. pallidum* but was *not* even reduced by rabbit testicle tissue. The interpretation of this would be that *the antigens present in tissues from different species are not immunologically identical* and *that the immobilizing* antibodies in different human NS have developed under the stimulus *of dissimilar* antigens. The fact that human antibodies were not absorbed by homologous tissues and that monkeys did not develop immobilizing antibodies after inoculation of homologous *tissues* lend further evidence to the theory according to which the immobilizing principle *of* NS possesses immunological specificity. The reason why monkey testicle tissues *failed to* absorb the immobilizing activity of human NS may be that absorption experiments *are highly* dependent on antigen concentration and antibody avidity and that the *sensitivity* of the inoculation procedure was higher than that of absorption for the demonstration of anti-

TABLE 2 *Development of* T pallidum *Immobilizing Activity of NS Type in the Cynomolgus Monkey upon Inoculation of* T pallidum *and various Tissues. The Immobilizing Serum Titre of each of the Monkeys prior to Inoculation was <2.0*

Inoculum	Monkey number	Immobilizing titre (IT_{50}) of NS type	
		One week after inoculation	Two weeks after inoculation
Killed *T pallidum*	I	6.2	8.2
	II	5 4	8.1
HeLa cells	I	<2 0	3 1
	II	<2 0	2.6
Rabbit testicle	I	4 1	8.2
	II	4 0	6.4
Rabbit liver	I	4.2	5 6
	II	2.2	4.9
Guinea pig testicle	I	2 6	4 0
	II	<2 0	5 8
White rat testicle	I	5 0	5.9
	II	2.8	6 2
Monkey testicle	I	<2 0	<2.0
	II	<2 0	<2.0

Development of T pallidum *Immobilizing Activity of the NS Type in the Cynomolgus Monkey Following Inoculation of* T pallidum *and Various Mammalian Tissues*

Cynomolgus monkeys lacking *T pallidum* immobilizing serum activity were inoculated with suspensions of *T pallidum* purified and killed by gradient centrifugation and intact HeLa cells. Furthermore homogenates of various animal tissues rabbit liver and testes from rabbit guinea pig white rat and monkey were used as inocula Each preparation was inoculated into two monkeys. One or two weeks after the inoculation unheated serum from each of the monkeys, with the exception of the two animals inoculated with monkey tissues, possessed *T pallidum* immobilizing activity (Table 2) Kinetically the immobilizing reaction caused by *these monkey sera* behaved like that caused by human NS and their immobilizing activity was abolished upon *absorption* with *T pallidum* at 0° C If sera were heated and tested in the TPI test, none of these were found to be reactive

Absorption of Normal Serum (NS) with T pallidum *and Various Mammalian Tissues*

The same tissues as those inoculated into monkeys were used for absorption of four human NS Upon absorption with *T pallidum* the immobilizing activity would be abolished. Absorption with monkey tissues or HeLa cells, intact or as a sonicate did not affect this serum activity *Testicular tissue* from white rat and guinea pig reduced the titres of all sera if rabbit testicles and rabbit liver were used, the titre would be reduced in three of the four sera (Table 3)

DISCUSSION

In preliminary experiments it was shown that *absorption with suspensions* of living *T pallidum* purified from rabbit testicle tissue by low speed centrifugation did not reduce significantly the TPI titre of syphilis serum (IS) or the immobilizing activity of normal serum (NS) It was suggested that the low

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DECREASED DELAYED HYPERSENSITIVITY TO TUBERCULIN DEMONSTRATED IN EXPERIMENTAL LEPTOSPIROSIS IN GUINEA PIGS

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Colding, H., Johansen, K. Stæhr & Bentzon, M. Weis. Decreased delayed hypersensitivity to tuberculin demonstrated in experimental leptospirosis in guinea pigs. Acta path. microbiol. scand. Sect. C, 84: 255–260, 1976.

Skin reactivity to tuberculin has been studied during the course of experimental leptospirosis in guinea pigs. A depression of the delayed hypersensitivity to tuberculin was demonstrated in the infected animals. The depression was most pronounced when icterus had developed. The depression was not correlated with the amount of infectious units administered or with the demonstration of live leptospirae in the peritoneal cavity. In the infected animals there was no correlation between the initial and the final skin tests which is in contrast to findings in the control group.

Key words: Decreased delayed hypersensitivity; experimental leptospirosis; tuberculin; guinea pig.

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A decreased cutaneous reaction to tuberculin has been observed in viral infections (8, 10) and after the administration of viral vaccines (7). Similar phenomenon have been observed in bacterial infections (1). To the best of our knowledge, the cellular immune response to tuberculin during leptospirosis has not been studied previously. In the present study tuberculin sensitized guinea pigs were infected with *Leptospira icterohaemorrhagiae* and examined for sensitivity to tuberculin during the infection.

MATERIALS AND METHODS

Animals

Outbred male guinea pigs from Statens Seruminstitut were used in all assays. The guinea pigs used in the preliminary study weighed 450 g, while the weight varied from 630 to 750 g in all other experiments.

Method

Guinea pigs were sensitized by four simultaneous intracutaneous injections of tubercle bacilli into the ventral skin which were carried out about 5 weeks prior to the experiments in the Tuberculin Department, Statens Seruminstitut. Each injection consisted of 0.1 ml of a suspension of heat-killed tubercle bacilli in paraffin oil (Marcol 52) containing 0.4 mg dry weight per ml. Initial skin tests were performed in the Tuberculin Department about

genicity In all probability, these various tissues and the treponemes contain cross reacting antigens which in our test system give rise to identically reacting antibodies.

The suggestion that rabbit tissues contain antigens cross-reacting with *T pallidum* is in agreement with the findings by *Julian et al* (1963) and *Miller et al* (1966) who observed that goats, upon prolonged immunization with rabbit testicular material, developed *T pallidum* immobilizing antibodies. In contrast to the immobilizing antibodies of NS those from the immunized goats were apparently detectable by the conventional TPI test.

The current view concerning the origin of naturally occurring antibodies such as the *T pallidum* immobilisins of NS is that they like all other forms of antibodies developed after stimulation of an antigen (*Boyden* 1966) The results obtained in the present work by inoculation into monkeys are in support of this standpoint In a previous work it was shown that umbilical cord sera usually lacked immobilisins (*Hederstedt* 1976 a) while the immobilizing activity in sera taken within one year after birth was about the same as that in adults (*Hederstedt* 1976 b) Since the antigens of the immobilisins are so widely distributed in nature antibody production is apparently induced very soon after birth by antigens in the food (*Sterzl et al* 1965) or by contact with the normal flora of saprophytic treponemes (*Deacon & Hunter* 1962)

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REFERENCES

1 *Boyden S V* Natural antibodies and the immune response In Dixon F J and Humphrey J H (Eds.) Advances in Immunology vol. 5 Academic Press, New York and London 1966 p 1–74

2 *Deacon W E & Hunter E F* Treponemal antigens as related to identification and syphilis serology (27515) Proc Soc. Exptl Biol Med. *110* 352–356 1962

3 *Gajdusek D C* An autoimmune reaction against human tissue antigens in certain acute and chronic diseases. A.M.A. Archives of Int Med. *101* 9–29 1958.

4 *Hederstedt B* Studies on the complement activity in the *Treponema pallidum* immobilization (TPI) test. A comparative study of the immobilizing and hemolytic complement activity Acta path. microbiol scand. *53* 180–190 1961

5 *Hederstedt B & Skog E.* Quantitative *Treponema pallidum* immobilization (TPI) test in early syphilis. Acta derm. venerol. *44* 82–90 1964

6. *Hederstedt B.* Studies on the *Treponema pallidum* immobilizing activity in normal human serum. I A method Acta path. microbiol. scand. Sect. B *82* 185–200 1974

7 *Hederstedt B* Studies on the *Treponema pallidum* immobilizing activity in normal human serum. 2. Serum factors participating in the normal serum immobilization reaction. Acta path. microbiol scand. Sect. C, *84* 135 141 1976 a.

8 *Hederstedt B* Studies on the *Treponema pallidum* immobilizing activity in normal human serum. 4 On the importance for the outcome of the conventional TPI-test. Acta path microbiol scand. Sect. C,*84* 148 152, 1976 b

9 *Hederstedt B* Studies on the *Treponema pallidum* immobilizing activity in normal human serum. 5 On the protective role against syphilis. Acta path. microbiol. scand. Sect. C, *84* 245–249 1976 c

10 *Julian A J Portnoy J & Bossak H W* False positive reactions in treponemal tests Brit J Vener Dis. *39* 30–32, 1963

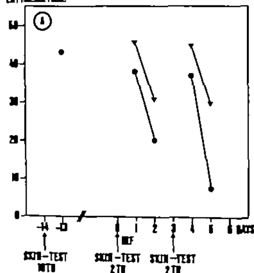
11 *Julian A J Logan L C & Norins L C.* Early syphilis immunoglobulins reactive in immunofluorescens and other serologic test. Brit J vener *39* 30–32, 1969

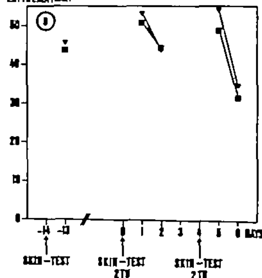
12 *Laurell A B. & Hederstedt B.* Fractionation of TPI antibodies and Wassermann reagins. Acta path microbiol scand. *44* 88 91 1958.

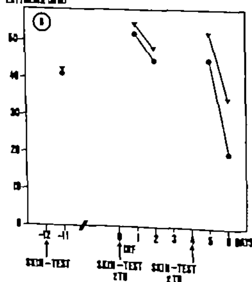
13 *Metzger M Michalska E Podwinska J & Smogór W* Immunogenic properties of the protein component of *Treponema pallidum* WHO/VDT/RES 69 165 1969

14 *Miller J N Bekker J H DeBruijn J H & Onvlee P C* The immunologic response of goats to normal and syphilitic rabbit testicular tissue J Immunol *97* 184–188, 1966

15 *Sterzl J Vesely J Jilek M & Mandel L.* Molecular and cellular basis of antibody formation. (J Sterzl Ed.) Czech Acad. Sci. Prague 1965 p. 463–476







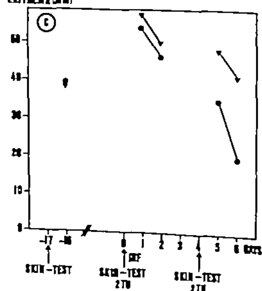


Fig. 1 A–D Average results of the skin tests in four different experiments.
■ Injected with Korthof medium
● Infected with *Leptospira icterohaemorrhagiae*
▼ Controls

2 weeks before infection with leptospirae Dilutions of purified tuberculin (PPD) RT 23 Statens Seruminstitut, were used The diameter of the erythemas were recorded by two persons 24 hours after infection (7)

The skin tests were repeated on the day of infection with leptospirae (*day 0*) and again on day 3 or day 4 using 0 1 ml of a dilution of purified tuberculin (PPD) RT 23 containing 2 tuberculin units per 0 1 ml Skin reactions were measured 24 and 48 hours after each test

The animals were infected intraperitoneally with 0.2 ml of a 3-4 weeks old culture of *L. icterohaemorrhagiae* obtained from infected mice or guinea pigs. The culture had grown in Korthof's medium for 14 days at 29 C and thereafter at 2?-25 C for 1-2 weeks. The cultures were kindly provided by Dr *C Borg Petersen* Statens Serum Institut. During the period of experimental infection the weight and clinical condition of the animals were recorded daily and the peritoneal exudate was examined microscopically for leptospirae on day 3 or day 5

In the preliminary study the guinea pigs were infected on three different days in groups of three and nine uninfected animals served as controls.

In experiment 1 eight animals were infected on the same day with 20-25 × 10⁸ viable organisms and eight uninfected animals served as controls. The animals were picked at random for the two groups.

In experiment 2 the procedure used in experiment 1 was followed except that the number of live leptospirae used for infection was only about half of that used in experiment 1

In the control experiment the procedure used in experiment 1 was followed, except that only 0 2 ml of Korthof's medium was used for i p injection.

Statistical Analysis

The statistical tests carried out are based on non parametric statistics. *Wilcoxon's* two sample test (11) is used for the pairwise comparisons in Table 1

The possible correlation between the results of the first and the final skin test is estimated by Spearman's (6) coefficient of rank correlation Since no such correlation could be demonstrated in the group of infected animals the first readings are not used as covariates in the comparisons.

RESULTS

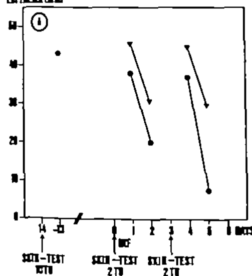
In the pilot experiment there was a difference in the skin tests in infected animals as compared with uninfected animals. The difference seems to be most pronounced 48 hours after the third skin test (Fig 1 A) This observation is confirmed by the results from experiments 1 and 2, as shown in Figs. 1 B and 1 C.

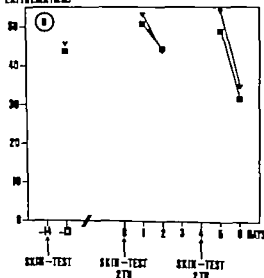
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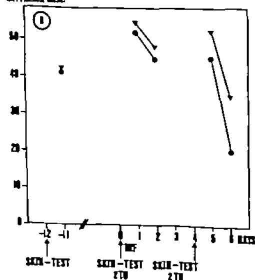
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Fig 2 shows the results of the third skin test performed on day 4 and read on day 6 (experiments 1 2 and control) The distribution of the diameters of the erythemas in the infected animals is at a lower level than that in the uninfected controls.

Two of the infected animals from experiment 2 were clinically unaffected even though live leptospirae were found in the peritoneal cavity Their skin reactions were within the same range as those of the controls. In experiment 1 one of the infected animals died on day 6 All the infected animals in experiments 1 and 2 lost in weight,







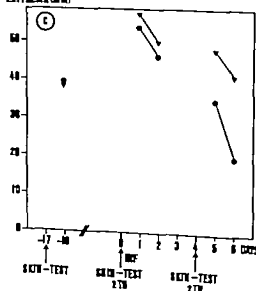


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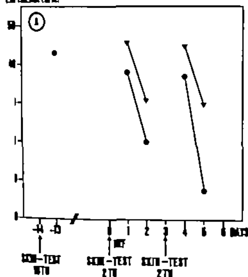
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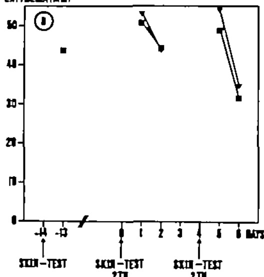
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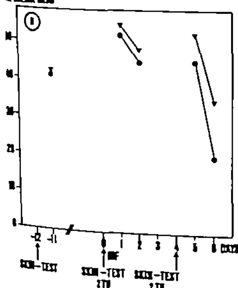
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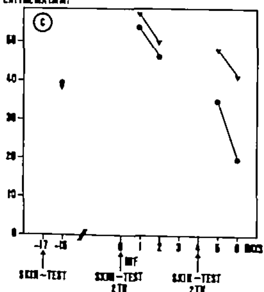


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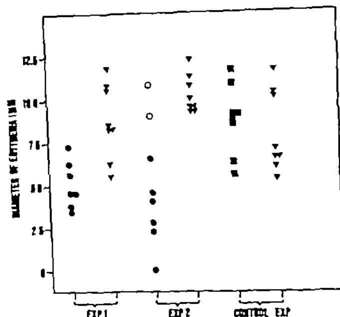


Fig 2 Results of skin tests in guinea pigs read on day 6.
● Infected with *Leptospira icterohaemorrhagiae*
○ Infected but unaffected
■ Injected with Korthof's medium
▼ Controls
One of the infected animals died on the 6th day of experiment 1 the diameter of the erythema was then 6 mm.

given was found. Our results suggest a non specific inhibition of skin reactivity to PPD A specific inhibition of T cell response to leptospiral antigens has not been described, although reduced T cell function, specific as well as unspecific, is reported to occur in other acute and chronic infections (9 12)

A possible explanation of anergy during infections has been discussed by *Kantor* (5) who states that lymphocyte products initiated by the immune response might suppress T cell function

Plasma inhibitors of lymphocyte responses have been observed in several diseases including another spirochete infection. Circulating lymphocyte inhibitors are most prominent at the height of the inflammatory reaction (4) This corresponds with our findings of increasing depression of skin tests in the acute leptospiral infection accordingly as the inflammation becomes more pronounced.

The nature of the inhibitors is not fully known. Antibodies to lymphokines have not been demonstrated *in vivo* but antibodies produced in rabbits have been reported to be able to abolish migration inhibitory factor chemotactic factor and skin reactivity to PPD in sensitized guinea pigs (3 13)

The different size of skin reactions to tuberculin may be explained by genetic variations among the animals and the different susceptibility to the same amount of infectious units may also be due to the heterogeneity of the group (4) The lack of correlation between tuberculin reactivity and immune response to leptospirosis suggests a genetic independence.

Further studies are needed to elucidate the nature, the specificity and the genetic relationship of the inhibition of T cell function during leptospirosis.

REFERENCES

1 *Bentzon J W.:* The effect of certain infectious diseases on tuberculin allergy Tubercle *34* 34 41 1953.
2. *Brody J A., Overfield T & Hammes L M.:* Depression of the tuberculin reaction by viral vaccines. New Engl. J Med. *271* 1294–1296, 1964
3. *Geczy C. L., Friedrich W & de Weck A L.* Production and in vivo effect of antibodies against guinea pig lymphokines. Cell. Immunol. *19* 65–77 1975.
4 *Green, I.* Genetic control of immune responses Immunogenetics *1* 4 21 1974

TABLE 1 *Statistical Comparison of the Diameters of Erythema in Infected and Uninfected Animals*

	Reading	Number of animals		Wilcoxon test		
		Inf	Control	"U"	T_1	P (%)
Pilot	1	9	9	−1.94	63.5	5–10
	2	9	9	−2.56	56.5	1–2
	3	9	9	−2.73	54.5	<1
	4	6	6	−1.68	28.5	>10
	5	2	6	−1.67	4	-
Exp 1	1	8	8	−1.00	58.5	>10
	2	8	8	−1.00	58.5	>10
	3	8	8	−1.37	55	>10
	4	8	8	−1.53	53.5	>10
	5	7	8	−2.78	32	<1
Exp 2	1	8	8	0.37	71.5	>10
	2	8	8	−1.21	56.5	>10
	3	8	8	−1.37	55	>10
	4	8	8	−2.99	39.5	<1
	5	8	8	−2.60	33.5	<1
Control exp	1	8	8	0.37	71.5	>10
	2	8	8	0.68	74.5	>10
	3	8	8	−0.37	64.5	>10
	4	8	8	1.21	79.5	>10
	5	8	8	0.57	73.5	>10

TABLE 2 *Test for Correlation Between Skin Tests Readings 1 and 5*

	Animal group	No. of animals	Rank correlation	P (%)
Exp 1	Infected	7	−0.12	>10
	Control	8	0.76	2–5
Exp 2	Infected	8	0.38	>10
	Control	8	0.45	>10
Control exp	Injected	8	0.49	>10
	Control	8	0.80	2–5

but the loss was not correlated with the results of the skin tests All were jaundiced on day 7 except the two that were clinically unaffected. The uninfected controls and those that had only received Korthof's medium either maintained their weight or gained in weight.

None of the infected animals in experiments 1 and 2 became clinically ill until the 5th day after the infection although live leptospirae were demonstrated in the peritoneal cavity

DISCUSSION

The present study has shown that there is a depression of delayed hypersensitivity to tuberculin in the acute state of leptospirosis in guinea pigs No correlation between skin reactivity and the amount of infectious units

Acta path. microbiol. scand. Sect. C, 84 261–269 1976

DETECTION AND DIFFERENTIATION OF IMMUNE COMPLEXES AND IgG AGGREGATES BY A COMPLEMENT CONSUMPTION ASSAY

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Nielsen, H & Svehag, S.-E. Detection and differentiation of immune complexes and IgG aggregates by complement consumption assay Acta path. microbiol. scand. Sect. C, 84 261 269 1976.

The applicability of a complement consumption assay as a means by which to detect IgG aggregates and immune complexes in serum was examined. Both heavy (≥19S) and intermediate (11 17S) IgG aggregates were detected and the sensitivity of the assay was ≥10 μg aggregated IgG/ml. BSA anti-BSA complexes, formed in slight antibody excess, were detected at a BSA concentration of 200 ng/ml. NHS stored at 4 C for ≥2–3 weeks or at –20° C for more than 3 months developed distinct anticomplementarity (AC) This background AC, due to IgG aggregate formation, was reduced by heating the serum at 56 C for 30 min prior to testing A similar reduction of AC and C1q fixation was observed when IgG aggregated at 61 C or 63 C was heated further at 56 C for 30 min The abatement of AC could not be correlated to change in IgG aggregation size. I contrast, AC of preformed antigen-antibody complexes was not reduced by this heat treatment.

Key words Immunecomplexes Ig-aggregates complement consumption assay

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It is well documented that immune complexes (IC) play an important pathogenic role in SLE and SLE-associated renal damage (17 25 30) In addition, IC-involvement is present in several human diseases of known aetiology such as acute poststreptococcal glomerulonephritis, hepatitis B-associated nephritis, malaria nephropathi and subacute bacterial endocarditis (4 6) Several chronic viral infections in animals are associated with IC-nephritis.

It is clearly desirable, in the diagnostic work, that the routine immunofluorescent techniques can be supplemented with simple and reproducible methods by which to demonstrate circulating IC in patients sera. In parallel with the development of sensitive radio-immuno-assays for the detection of circulating IC (5 7 26, 27) we have explored also whether a complement consumption test might be of use for the same purpose. The application of this technique and its sensitivity for the detection and characterization of Ig aggregates in human sera, and its potential usefulness for the demonstration of IC in

5 *Kantor F S* Infection, anergy and cell mediated immunity New Engl. J Med. *292* 629–634 1975
6 *Kendal M G* Rank Correlation Methods, 2nd Ed., London 1955 See also Documenta Geigy Scientific Tables, 6th Ed., p 180 19B
7 *Kim H K Magnusson M & Bentzon M W* Tuberculin production 2 Comparison between amount of tuberculin obtained from the culture filtrate and by extraction of tu bercle bacilli. Acta path microbiol scand *58* 501–510 1963
8 *Reed W P Olds J W & Kirsch A L.* Decreased skin test reactivity associated with influenza J infect. Dis. *125* 398–402 1972.
9 *Turk J L.* Immunological spectra in chronic infectious diseases. Boll Ist. sieroter mila. *53* 208–212 1974
10 *Von Pirquet C* Das Verhalten der kutanen Tuberkulin Reaktion während der Masern. Dtsch. med Wschr *34* 1297–1300 1908.
11 *Wilcoxon F* Individual comparisons by rank ing methods. Biometrics *1* 80–83 1945. See also Documenta Geigy Scientific Tables, 6th Ed., p 191 24B.
12 Wld Hlth Org techn, Rep. Ser No. 519 p. 45–49 1973: Cell mediated immunity and resistance to infection. Report of a scientific group Geneva 1973
13 *Yoshida, T Bigazzi P E. & Cohen S* The production of anti-guinea pig lymphokine antibody J Immunol. *114* 688–691 1975.

in 0.2 ml veronal buffer and 0.2 ml rabbit anti-BSA (Behringwerke A G Germany) diluted 1:4 in the same buffer were mixed and incubated at 22° C for 45 min. The molar antigen-antibody ratio was estimated to be about 2 1 by reference to quantitative precipitation curve.

Gel filtration chromatography Two ml Δ_{61} IgG 20 mg/ml, was filtered on Sephadex G 200 column (2.6 × 100 cm, Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0 01 M Tris-HCl buffer containing 0.5 M NaCl and 0 02 per cent NaN_3, pH 8.0. The column was run at 4 C and the flow rate was 5 ml/hour Selected fractions were collected and examined for anti-complementarity by the complement consumption (CC) test.

Five ml of NHS pool, stored at −20° C for 12 months, was filtered on an Ultrogel AcA 22 column (2 6 × 100 cm, LKB, Stockholm, Sweden) The column was kept at 4 C, equilibrated by the Tris-HCl buffer containing 0.5 M NaCl and the flow rate was 5 ml/hour Eluted fractions were analysed by the CC-test.

C3PA conversion was determined by an immunoelectrophoresis technique in which formation of C3 activator was estimated (13) after treatment of serum with aggregated human myeloma IgA (kindly supplied by Dr. *P Brandtzaeg*) *E. coli* LPS and zymosan Analyses were performed in 1 per cent agarose gel using sodium diemal buffer pH 8 6 ionic strength 0 02 containing 0.01 M EDTA and a potential gradient 5 V/cm for 2 h at room temperature. Anti C3PA/C3A serum (Behringwerke AG Marburg, Germany) was diluted 1/10 and added to the through.

Fused rocket immuno-electrophoresis (28) Four µl of the material to be tested was pipetted into wells and allowed to diffuse at 22 C for 45 min in 1 per cent agarose gel (Indubiose A37 L'Industrie Biologique Française S.A.) This gel was then placed in close contact with 1 per cent antiserumcontaining gel and electrophoresis was performed at 2.5 V/cm for 20 hours. The distance between the wells and the antibody-containing gel was 1 cm. The same veronal buffer μ = 0 002, pH 8 6 was used in the agarose and the vessels. Antisera to IgG and IgM were purchased from Dakopatts A/S, Copenhagen, Denmark The plates were dried and stained with 0.5 per cent Coomassie brilliant blue R 250 according to standard procedures

RESULTS

Effect of storage temperature and heat treatment on the anticomplementarity of normal human sera Normal donor sera as well as patient sera used in the present study were stored at −20° C in small aliquots. Repeated

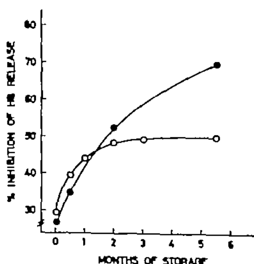


Fig 2 Formation of anticomplementary (AC) activity in NHS samples during storage at −20° C for up to 5½ months. AC was determined as per cent inhibition of haemoglobin release in the complement consumption assay Samples heated at 56 C for 30 min just prior to testing ○ samples not heated ●

freezing of specimens was avoided. Fig. 2 illustrates the formation of anticomplementary (AC) activity in serum samples collected from one and the same donor and stored at −20 C for variable times. The samples were tested in parallel on the same day A continuous increase in AC was observed during storage at this temperature. All specimens had the same very low AC when tested fresh, prior to storage. The same specimens were tested also after heat treatment (56 C for 30 min) just before performing the CC-test. The effect of heat treatment on the AC of the sera differed for sera with pre-existing high and low complement consuming activity Heat treatment caused a slight increase of AC in sera stored ≤2 months (Figs. 2 & 3) while the same treatment reduced the strong AC developed in sera stored over long periods (Fig. 2) Serum samples kept at 4 C showed quite strong AC—up to 90 per cent inhibition of haemoglobin release—already after 2 to 3 weeks' storage (Fig. 3) Subsequent freezing of the specimens at −20 C or −60° C did not affect this high AC level. Heat treat

patient sera, are reported in this communication In addition the effect of storage temperatures and heat treatment on the formation of anticomplementary activity in human sera and IgG preparations, was examined

MATERIALS AND METHODS

Complement Fresh guinea pig serum was absorbed with sheep erythrocyte (SE) stroma. Nine parts serum and 1 part packed washed stroma were incubated at 4 C for 15 min and the stroma was removed by centrifugation at 3000 × *g* for 10 min Absorbed serum was stored at –70 C. The complement titre was 64–128.

Haemolysin Rabbits were immunized twice by s.c. injections of 0.5 ml 5 per cent SE at multiple sites followed by three i.v booster injections with SE stroma at 3 days intervals. The animals were bled 10 days after the last i v injection the serum was collected, heated at 56 C for 30 min and stored at –60 C. The haemolysin titre was around 40 000

Sheep erythrocytes One volume sheep blood was collected in 1 volume Alsever s solution pH 6 1 and kept at 4 C for a maximal period of 3 weeks. The erythrocytes were washed 3 times in physiological NaCl before use. A 4 per cent erythrocyte suspension was used in the complement consumption test.

Complement consumption assay 150 µl sample to be tested, non heated or heated at 56 C for 30 min was mixed with 20 µl guinea pig serum (pool) and incubated at 37 C for 30 min. The mixture (100 µl) was exposed to two-fold titration in veronal buffer 100 µl of a 4 per cent suspension of SE washed after optimal sensitization with 16–32 haemolysing units at 37 C for 30 min was added to each tube the tubes were shaken and incubated at 37 C for a further 30 min Finally 1 ml cold PBS buffer was pipetted into each tube, the tubes were centrifuged at 3,000 × *g* for 5 min and the haemoglobin release in the supernatants was determined spectrophotometrically at 545 nm. As a positive control heat treated (63 C for 12 min or 61 C for 40 min) human IgG preparation was used at a concentration of 20 µg/ml and 150 µg/ml veronal buffer respectively Veronal buffer was used as a reference CH_{50} and normal human sera (NHS) free of heterophilic antibodies against the target cells, as negative controls. The veronal buffer (pH 7 4) used had the following composition diemal sodium 10.2 g NaCl 88.9 g $CaCl_2$ 85 mg, $MgCl_2$ 500 mg 1 M HCl 31.3 ml in 2 l dist. H_2O The buffer was diluted 1 5 in dist. H_2O prior to use.

A typical test result of the standard experimental design is seen in Fig. 1 The negative NHS controls usually gave a slightly enhanced haemoglobin release (here to 53 per cent) as compared to the CH_{50} reference value obtained by veronal buffer while the positive control sample ($▲_{61}$ IgG) caused about 80 per cent inhibition of the CH_{50} release The anticomplementary (AC) activity of the various IgG specimens and human sera examined is expressed as per cent inhibition of the CH_{50} release. The background haemoglobin release in the absence of complement or haemolysin at this titration point (1 8) was about 2 per cent.

The variance in the system at the CH_{50} reference level was statistically evaluated on the basis of a negative control (NHS) sample tested 20 times over a 3-month-period using triplicate determinations in one serie and single determinations in two series. During this period of time the anti erythrocyte serum as well as the complement and the sheep erythrocytes were exchanged. The SD was 3 4 per cent and the SD_b = 1.9 per cent. Error of mean based on the triplicate determinations was estimated to be $SE_{\bar{x}}$ = 2 8 per cent.

Preparation of aggregated human IgG by heating Aggregated IgG was prepared by heating 20 mg human IgG (Cohn s fraction II and III Kabi, Stockholm, Sweden) in 1 ml veronal buffer at 61 C for 40 min (Δ IgG) or at 63 C for 12 min ($Δ_{63}$ IgG)

Preparation of albumin anti-albumin complexes. BSA (Behringwerke A.G Germany) 100 µg/ml

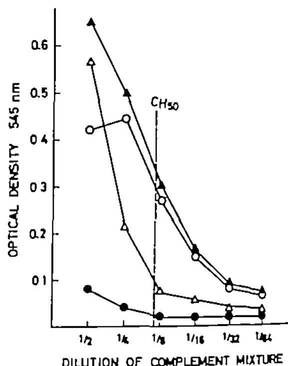


Fig 1 Typical results obtained by the standard design used in the complement consumption assay Veronal buffer ○ negative NHS control ▲ positive control, $Δ_{61}$ IgG Δ and background haemoglobin release ●

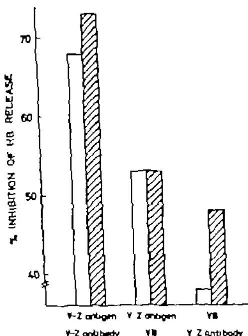


Fig 5 Effect of heat treatment on the anticomplementarity of varicella-zoster antigen-antibody complexes. Hatched bars indicate samples heated at 56 C for 30 min before testing.

ficant AC would be detected at a concentration of 10 to 20 μg/ml.

The anticomplementarity of BSA anti-BSA complexes formed at equilibrium and antibody or antigen excess was examined also. It appears from a comparison with the quantitative precipitation curve that maximal anticomplementarity was recorded in the region of slight antibody excess, corresponding to a molar ratio of BSA to anti-BSA IgG of 2 1 (Fig 7). The lowest BSA concentration contributing to the complex formation at this molar ratio 200 ng/ml. There was no observable effect of heat treatment (56 C for 30 min) on the AC of BSA anti BSA complexes at this concentration of the immune complexes.

Size of IgG-aggregate detectable in test sera. A Δ_{40} IgG solution (20 mg/ml) was fractionated on a Sephadex G 200 column. The major part (about 63 per cent) of the 7S IgG preparation had been converted to

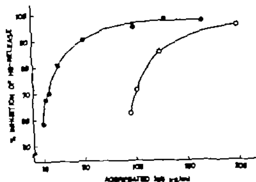


Fig 6 Sensitivity of the CC-assay for the detection of heat aggregated IgG. The anticomplementarity of two IgG preparations, Δ IgG (40 min) ○ and Δ_{63} IgG (12 min) ● was determined at concentrations from 10–200 μg/ml.

19S or slightly larger aggregates (Fig. 8). The aggregated IgG showed strong AC-activity in the CC-test, while no AC was registered in any other fractions on the elution profile. There was no indication that intermediate size aggregates were formed by this heat treatment.

Long term storage of IgG or NHS causes the formation of intermediate size (11–17S) aggregates (7). In order to ascertain whether the CC-technique would detect such aggre

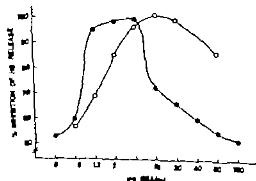


Fig 7 Anticomplementarity of BSA anti-BSA complexes ● at different regions of the quantitative precipitation curve ○. Maximal anticomplementarity was recorded in slight antibody excess, corresponding to a molar ratio of BSA to anti-BSA IgG of 2 1

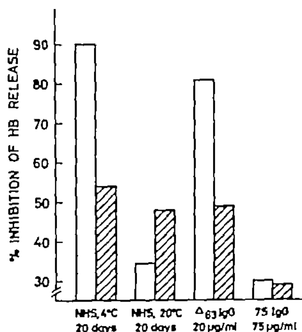


Fig 3 Effect of heat treatment on the anticomplementarity of NHS stored at 4 C for 20 days, NHS stored at −20 C for 20 days, human IgG aggregated at 63 C for 12 min and 7S IgG respectively Hatched bars indicate samples heated at 56 C for 50 min just before testing

ment (56° C for 50 min) of the serum samples stored at 4 C, just before testing in CC reduced the AC by roughly 40 per cent (Fig 3)

Effect of heat treatment on the anticomplementarity of human IgG and antigen antibody complexes Human Δ_{63} IgG exerted strong AC if tested by CC at a concentration of 20 μg/ml veronal buffer (Fig 3) Similar to the findings previously obtained in the studies of NHS this strong AC could be reduced distinctly by a further heat treatment (56° C for 50 min) just before the specimens were assayed in the CC-test This effect could be related to a reduced capacity of the binding of the IgG to human C1q (23) The unheated original 7S IgG preparation had no AC activity if tested at a concentration of 75 μg/ml. Complexes of BSA (5 μg/ml) and rabbit anti BSA IgG antibodies were formed at equilibrium. The AC activity of the preformed immune complexes was determined before and after heating at 56 C for 50 min. No significant change in anticomplementariness was observed after this heat treatment (Fig 4) The same results were obtained in a system more *in vivo* related in which serum from a patient in an early phase of varicella zoster disease (CF antibody titre 32) was used The serum sample was incubated with varicella zoster antigen and generated AC activity was determined before and after heating of the mixture at 56° C for 50 min. No decrease in anticomplementariness could be demonstrated (Fig 5) after the heat treatment.

Sensitivity of complement consumption assay for the detection of heat aggregated IgG and immune complexes The AC-activity of human IgG increased with time of heating at 61° C reaching maximum after approximately 40 min. Further heating at this temperature slightly reduced the AC of the IgG preparation. Different amounts of Δ_{61} IgG heated for 40 min were examined for AC-activity by the CC-test. Significant AC was detected at concentrations of Δ_{61} IgC equal to or above 80 μg/ml (Fig 6) If the same 7S IgG preparation, heated at 63° C for 12 min (Δ_{63} IgG) was tested in parallel, signi

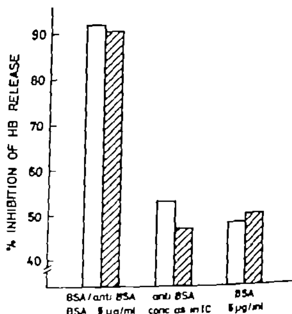


Fig 4 Effect of heat treatment on the anticomplementarity of preformed BSA anti BSA complexes. The molar ratio of antigen to antibody was 2 1 and the antigen concentration was 5 μg/ml Hatched bars indicate samples heated at 56 C for 50 min before testing

CC-method, in parallel with radio-immuno-assays (8, 23) have indicated, however that the method registered circulating IC in most cases where sensitive RIA-techniques indicated its presence.

The present assay detected IgG aggregates of both intermediate sizes and sizes ≥19S. By way of comparison, the C1q-precipitation technique and the platelet aggregation method detect only large complexes (≥19S)

In contrast to some of the above mentioned alternative techniques (5 7 26, 27) for IC detection, based on C1q-binding or -precipitation, a CC-assay should theoretically be able to detect inducing factors involved in both classical and alternative pathway (10 13 22) activation of the C-system. The classical pathway can be activated by aggregated IgM and IgG (subclasses 1 2 and 3) or IC containing these immunoglobulins (14) Aggregated secretory IgA has been reported to activate the C-system over the alternative pathway (3) and aggregated human myeloma IgA to convert C3PA to C3A (29) We were unable to demonstrate either complement consumption or a distinct C3PA-conversion after addition of an aggregated (heat treated or freeze dried) human myeloma IgA preparation (≤600 μg/ml) to whole human serum. As regards the capacity of aggregated IgA to activate the C-system, conflicting results have been obtained in other studies of different IgA myeloma proteins (3 13 14 20)

The formation of Ig-aggregates during handling and storage of patient sera presents a potentially disturbing source of error in determinations of circulating IC, particularly in work with highly sensitive techniques. We have observed that NHS stored at 4 C for 2 3 weeks or at -20 C for more than 3 months developed rather strong AC-activity and distinct conversion of C3PA (24) The triggering of the classical complement pathway by formed IgG-aggregates generates C3b which in turn, particularly in the presence of a partial deficiency in C3b inhibitor may be responsible for an activation of the alternative pathway (18, 22) This explanation, however appears less likely in the present situation as no distinct C3PA conversion was demonstrable even after incubation of NHS with high concentrations (2 mg/ml) of aggregated IgG which effectively activated the classical pathway (24) Alternatively the possibility that serum enzymes slowly splits C3PA during storage should be considered, if however NHS which had developed strong AC during storage, were heated at 56 C for 50 min just before performance of the CC-test, the AC would be much reduced. The same effect of heat treatment on AC was described by *Johnson et al.* (16) and *Fromshagen & Fudenberg* (11) reported reduced background AC after heating of whole serum at 56 C for 60 min or 63 C for 20 min. Heating of purified 7S γ-globulin at 61–63 C for 10–20 min causes the formation of C-fixing IgG aggregates. If these heat aggregated IgG preparations were heated further at 56 C for 50 min just prior to the CC-test, the AC-activity would be reduced by 40 to 50 per cent (Fig 3) In addition, this treatment lowered the C1q-binding capacity of the IgG preparations, when examined in a parallel RIA-technique (27) There was no observable difference in the IgG aggregate distribution on a Sephadex G 200 column before and after this additional heating of the preparations. The prolonged heat treatment would apparently alter the IgG aggregates formed in NHS during storage or in 7S γ-globulin by a short heat pulse and thus, the C1q-binding sites would become less accessible or partly destroyed. In contrast, heating of preformed antigen-antibody complexes (BSA anti-BSA or varicella zoster antigen-antibody) in NHS at 56 C for 50 min did not affect their AC-activity These findings indicated that the background AC in stored patient sera could be reduced in the routine CC-assay by heating of the sera at 56 C for 50 min and still permit detection of IC in the same sera. An undesirable contribution of human C to the test system was eliminated simultaneously Finally it should be recognized that biologic polyanions such as DNA or endotoxin lipopolysaccharides present in certain patient sera

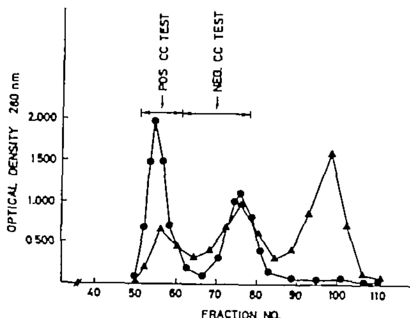


Fig 8 Anticomplementarity of Δ_{63} IgG fractionated on a Sephadex G 200 column. Fractions were tested at a concentration of 12 µg protein/ml Δ_{63} IgG ● NHS reference curve ▲

gates, a normal human serum sample, stored at −20° C for 1 year was examined also The serum sample was fractionated by gel-filtration on Ultrogel AcA 22 and eluted fractions were assayed in the CC-test (Fig 9) The presence of IgG and IgM in the fractions was indicated by the fused rocket electrophoresis technique. Moderate to strong AC was registered from the 19S peak down to the 10–11S region No AC was detectable in any other fractions.

DISCUSSION

The sensitivity of the described complement consumption assay based on the detection of 10–20 µg Δ_{63} IgG/ml, exceeds that of the Clq precipitation technique (1 2) by approximately 10 times. The platelet aggregation technique, however was reported to detect ≥2 µg Δ_{63} IgG (21) and the radioimmuno-assays recently developed (5 7 26, 27) appeared to be 10–100 times more sensitive. Studies of clinical specimens using the

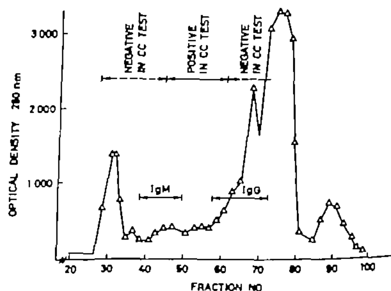


Fig 9 Anticomplementarity of NHS fractionated on an Ultrogel AcA 22 column, after storage at −20 C for 1 year The presence of IgM and IgG in the fractions was determined by fused rocket eletcrophoresis technique.

immune-electrophoresis technique for quantitation of C3PA conversion (Unpublished work)

25. *Onyewotu, I. I., Holborow E. J. & Johnson G. D.* Detection and radioassay of soluble circulating immune complexes using guinea pig peritoneal exudate cells. Nature *248* 156–159, 1974

26. *Sobel, A. T. Bokisch, V. A. & Müller-Eberhard H. J.* C1q deviation test for the detection of immune complexes, aggregates of IgG and bacterial products in human serum. J Exp. Med. *142* 139–150 1975

27. *Svehag, S.-E.*, A solid phase radioimmune assay for C1q-binding immune complexes. I. ΔIgG as indicator molecule. J Immunol. *4* 687–697 1975.

28. *Svendsen, P. J.*, Fused rocket immunoelectrophoresis. Scand. J Immunol., Suppl. 1 69–70 1973.

29. *Spiegelberg, H. & Götze O.* Conversion of C3 pro-activator and activation of the alternate pathway of human immunoglobulins. Fed. Proc. *31*: 655 Abs, 1972.

30. *Theofilopoulos, A. N., Wilson C. B., Bokisch V. A. & Dixon, F. J.* Binding of soluble immune complexes to human lymphoblastoid cells. J Exp. Med. *140* 1230–1244 1974

can bind C1q-protein and give false positive results in studies of circulating IC by C1q fixation or CC-techniques (2 15 19) The presence of endotoxin in patient sera, particularly of those derived from smooth strains of gram negative bacteria may also give positive results in the CC-test, due to their capacity to activate the alternative complement pathway (9 12 15)

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REFERENCES

1 *Agnello V Winchester R J & Kunkel H G* Precipitin reactions of the C1q component of complement with aggregated γ-globulin and immune complexes in gel diffusion Immunology *19* 909–919 1970

2 *Agnello V Carr R I Koffler D & Kunkel H G* Gel diffusion reactions of C1q with aggregated globulin, DNA and other anionic substances. Fed. Proc. *28* 2447 1969

3 *Boackle R J Pruitt K M & Mestecky J* The interactions of human complement with interfacially aggregated preparation of human secretory IgA. Immunochemistry *11* 543–548 1974

4 *Cochrane C G & Koffler D* Immune complex disease in experimental animals and man. Advan. Immunol *16* 185–264 1973

5 *Cowdery J S J Treadwell P E & Fritz R B.* A radioimmuneassay for human antigen antibody complexes in clinical material J Immunol *114* 5-9 1975

6 *Dixon F J* Pathogenesis of immunologic disease J Immunol *109* 187–192 1972

7 *Farrell C Søgaard H & Svehag S E* Detection of IgG aggregates or immune complexes using solid phase C1q and protein A rich *Staphylococcus aureus* as an indicator system Scand J Immunol. *4* 673–680 1975

8 *Farrell C Nielsen H Daugharty H Bloth B & Svehag S E* Soluble immune complex formation after myocardial infarction (in preparation)

9 *Fine D P Marney S R Colley D G & DesPrez R M* Decomplementation pattern in chelated and non-chelated serum. In Sell, S (Ed.) *Hemophilus influenzae* Proceedings of conference on antigen-antibody systems epidemiology and immunprophylaxis, Vanderbilt Press, Nashville Tenn., *1973* p. 113–117

10 *Frank M M & Gaither T.* The effect of temperature on the reactivity of guinea-pig complement with γG and γM haemolytic antibodies. Immunology *19* 967–974 1970

11 *Frommhagen L. H & Fudenberg H* The role of aggregated γ-globulin in the anticomplementary activity of human and animal sera. J Immunol *89* 336–343 1962

12. *Gewurz H Shin H S & Mergenhagen S E* Interactions of the complement system with endotoxic lipopolysaccharide Consumption of each of the six terminal complement components. J Exp. Med. *128* 1049–1057 1968.

13 *Götze O & Müller Eberhard H J.* The C3-activator An alternate pathway of complement activation. J Exp Med. *134* 90–108, 1971

14 *Ishizaka T Ishizaka K Salmon S & Fudenberg H* Biologic activities of aggregated γ-globulin. VIII Aggregated immunoglobulins of different classes. J Immunol *99* 82–91 1967

15 *Johnson U & Laurell, A B* Complement activation in meningococcal septicemia. Acta path. microbiol. scand. Sect. C, *83* 285–288, 1975

16 *Johnson H Mowbray J F & Porter K A* Detection of circulating immune complexes in pathological human sera. Lancet ii: 762–765, 1975

17 *Koffler D Agnello V Thoburn, R & Kunkel H G* Systemic lupus erythematosus prototype of immune complex nephritis in man. J Exp. Med *134* 169–179 1971

18. *Lachmann P J & Nicol P A E.* Studies on the C3b feedback cycle Adv in biosciences *12* 262–269 1973

19 *Loos M Bitter-Suermann D & Dierich, M* Interaction of the first (C1) the second (C2) and the fourth (C4) component of complement with different preparations of bacterial lipopolysaccharides and with lipid A. J Immunol *112* 935–940 1974

20 *Morse H* The aggregation of γ-myeloma proteins. J Immunol *95* 722–729 1965

21 *Myllylä G* Aggregation of human blood platelets by immune complexes in the sedimentation pattern test. Scand. J Hematol Suppl. *19* *1973*

22 *Müller Eberhard H J & Götze O* C3 proactivator con ertase and its mode of action. J Exp Med. *135* 1003–1008, 1972

23 *Nielsen H Ølholm P Daugharty H & Svehag S E* Detection and kinetics of circulating immune complexes in herpes zoster sera as revealed by three different assays. Abstract, European Immunology Meeting Amsterdam 1975

24 *Nielsen H & Svehag, S E.* Modified rocket

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Recently cell-mediated immunity (CMI) and humoral mediated immunity (HMI) against adult human oral mucosa (AHOM) and streptococcal antigens of *Streptococcus sanguis* strain 2A and *Streptococcus pyogenes* strain M5 have been demonstrated in patients with recurrent aphthous stomatitis (RAS) (5 7 8) This was shown by the leucocyte migration test (LMT) and the immunofluorescence (IF) technique, and it supported previous studies suggesting that auto-immunity and streptococcal hypersensitivity are involved in the pathogenesis of RAS (1 2, 3 4 6 9 11 12, 13 14 15 16, 17 18)

Only a few of the immunological studies have dealt with the CMI and the HMI against human oral mucosa in relation to the clinical activity of RAS These studies have indicated that CMI but not HMI against human oral mucosa are correlated with the activity of RAS (13 15 16)

The streptococcal hypersensitivity in relation to activity of RAS has, to the knowledge of the present author only indirectly been investigated in the study by *Sallay et al.* (17) These authors used Bencard bacteria antigen suspensions and found that the number and strength of positive skin reactions rose with the frequency of recurrences of RAS.

The aims of the present investigation were (a) to study the CMI and HMI against streptococcal and adult human oral mucosa (AHOM) antigens in relation to the clinical activity of RAS (b) to establish whether there is a correlation between the endpoint titres (EPT) and the migration indices (MI) against streptococcal and AHOM antigens in RAS (c) to determine the immunoglobulin classes of detected antibodies against streptococcal and AHOM antigens, and (d) to study whether antibodies involved were able to bind serum complement (C3)

MATERIAL AND METHODS

The present observations were obtained in cross-sectional and longitudinal series of investigation.

The series studied in the cross-sectional part of the investigation was composed of the groups of controls and patients with RAS presented elsewhere (5) Serum was collected together with blood samples for the LMT experiments and stored at −20° C.

In the longitudinal part of the study the series comprised 6 persons (3 females, age 22 52, average 38 3 males, age 19–69 average 39) without a past history of or clinical evidence of RAS and 7 patients (4 females, age 17–44, average 37 3 males, age 19–31 average 26) with RAS. The blood samples from the controls were collected at intervals of one week and those from the patients with RAS in relation to exacerbation of RAS The CMI and HMI were tested by the leucocyte migration test (LMT) and double layer immunofluorescence (IF) technique according to previous descriptions (5 6, 7 8)

The antigens used were adult human oral mucosa (AHOM) *Streptococcus sanguis* strain 2A, *Streptococcus pyogenes* strain M5 kidney and uterus (5) The IF experiments were carried out using AHOM, Strep. 2A, and Strep. M5 prepared according to *Donatsky & Dabelsteen* (7 8) The LMT experiments in the cross-sectional part of the study were carried out using tissue extracts of AHOM and streptococcal extracts according to *Donatsky* (5) In the longitudinal part of the study tissue extracts of AHOM, kidney and uterus were prepared according to *Donatsky* (5) The streptococcal suspensions were whole bacteria prepared according to *Donatsky & Bendixen* (6) The only reason why suspensions of whole streptococcal cells were used was that the longitudinal part of the present study was carried out in a period before the information on the effect of the disintegration by sonication had been obtained (5) In preliminary LMT experiments the highest nontoxic concentrations of the above antigens were determined and found to be as follows

Adult human oral mucosa (AHOM)	250 μg protein per ml
Fetal human kidney	100 μg protein per ml
Human uterus	250 μg protein per ml
Strep. 2A extract	20 μg protein per ml
Strep. 2A heat killed bacteria	1×10^8 bacteria per ml
Strep. M5 extract	2 μg protein per ml
Strep M5 heat killed bacteria	1×10^8 bacteria per ml

The above antigen concentrations were used as routine in the present investigations.

RESULTS

The results of the cross-sectional part of the study are presented in Figs. 1 2, 3 4 5 and in Tables 1 2, 3 The results of the longitudi

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COMPARISON OF CELLULAR AND HUMORAL IMMUNITY AGAINST STREPTOCOCCAL AND ADULT HUMAN ORAL MUCOSA ANTIGENS IN RELATION TO EXACERBATION OF RECURRENT APHTHOUS STOMATITIS

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Donatsky O Comparison of cellular and humoral immunity against streptococcal and adult human oral mucosa antigens in relation to exacerbation of recurrent aphthous stomatitis. Acta path. microbiol scand Sect C, *84* 270–282 1976

Cell mediated immunity (CMI) and humoral mediated immunity (HMI) against streptococcal and adult human oral mucosa (AHOM) antigens were investigated in patients with inactive and active recurrent aphthous stomatitis (RAS) The CMI and HMI were examined by means of the leucocyte migration test (LMT) and a double layer immunofluorescence (IF) technique The CMI against *Streptococcus sanguis* strain 2A, *Streptococcus pyogenes* strain M5 and AHOM was significantly increased in inactive and active RAS Significant difference between active and inactive RAS was only demonstrated when Strep. 2A was used as antigen in the leucocyte migration cultures. Longitudinal observations in relation to exacerbation of RAS revealed significant changes in the migration indices (MI) when Strep 2A and AHOM were used as antigens. Extracts of Strep M5 and other tissues as kidney and uterus did not show any significant changes of MI in relation to the exacerbation of RAS The IF investigations detected significantly raised IgG antibodies against washed whole cells of *Streptococcus sanguis* strain 2A and *Streptococcus pyogenes* strain M5 in sera from patients with RAS. The autoantibodies in RAS belonged to the immunoglobulin classes IgG and IgM The detected antibodies were able to bind serum complement (C3) The distribution of endpoint titres (EPT) against Strep 2A, Strep. M5 and AHOM antigens did not differ significantly in patients with active RAS in relation to those with inactive RAS Furthermore, the EPT against Strep. 2A and AHOM were not significantly changed in relation to exacerbation of RAS The present investigations show that CMI and HMI against streptococcal and AHOM antigens are characteristic features of RAS and indicate that the CMI against Strep 2A and AHOM is significantly increased in relation to exacerbation of RAS

Key words Recurrent aphthous stomatitis cell-mediated immunity humoral immunity leucocyte migration test immunofluorescence technique

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TABLE 1 *The Distribution of Endpoint Titres (EPT) of the Different Immunoglobulins against Strep. 2A in 23 Controls and 24 Patients with Recurrent Aphthous Stomatitis (RAS). Sixteen of These Patients Were in a Period with Remission and Eight Were in a Period with Exacerbation of RAS*

Strep. 2A	Immuno-globulin class	Endpoint titres 0	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Controls	IgG	1	0	4	10	4	3	1	0	0
N = 23	IgM	12	3	6	2	0	0	0	0	0
	IgA	8	9	4	2	0	0	0	0	0
Recurrent aphthous stomatitis	IgG	0	0	5	0	6	2	6	4	1
N = 24	IgM	11	9	1	1	2	0	0	0	0
	IgA	6	6	7	4	1	0	0	0	0
Recurrent aphthous stomatitis in period of remission	IgG	0	0	3	0	3	2	4	3	1
N = 16	IgM	7	7	0	1	1	0	0	0	0
	IgA	4	4	4	3	1	0	0	0	0
Recurrent aphthous stomatitis in period of exacerbation	IgG	0	0	2	0	3	0	2	1	0
N = 8	IgM	4	2	1	0	1	0	0	0	0
	IgA	2	2	3	1	0	0	0	0	0

and in patients with RAS is shown in Table 1. Immunoglobulins against Strep. 2A were detected in sera from patients with RAS as well as in sera from controls. The antibodies belong to the immunoglobulin classes IgG, IgM, and IgA. The IgG endpoint titres (EPT) against Strep. 2A were significantly higher in the group of patients with RAS than in the controls (Mann Whitney U-test, $p = 0.0043$). The distribution of IgM or IgA endpoint titres (EPT) against Strep. 2A in sera from RAS did not differ significantly from the distributions in the sera from controls ($p > 10$, $p > 10$). Furthermore, the statistical analysis

TABLE 2. *The Distribution of Endpoint Titres (EPT) of the Different Immunoglobulins against Strep. M5 in 16 Controls and 13 Patients with Recurrent Aphthous Stomatitis (RAS). Seven of These Patients Were in a Period with Remission and Six Were in a Period with Exacerbation of RAS*

Strep. M5	Immuno-globulin class	Endpoint titres 0	1:1	1:2	1:4	1:8	1:16	1:32
Controls	IgG	3	1	3	6	2	1	0
N = 16	IgM	15	1	0	0	0	0	0
	IgA	15	1	0	0	0	0	0
Recurrent aphthous stomatitis	IgG	0	0	3	3	2	3	2
N = 13	IgM	13	0	0	0	0	0	0
	IgA	12	0	1	0	0	0	0
Recurrent aphthous stomatitis in period of remission N = 7	IgG	0	0	3	0	1	2	1
Recurrent aphthous stomatitis in period of exacerbation N = 6	IgG	0	0	0	3	1	1	1

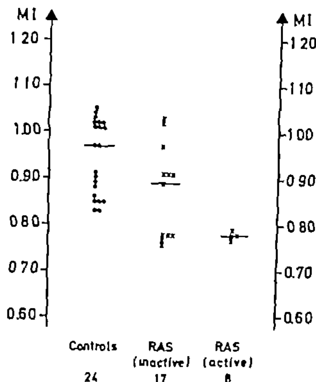


Fig. 1. Leucocyte migration test (LMT) with Strep 2A (20 µg protein per ml) showing the *migration indices (MI) of 24 normal controls,* 17 patients with inactive recurrent aphthous stomatitis (RAS) and 8 patients with active RAS.

nal part of the study are presented in Fig. 6 and in Table 4.

Fig. 1 shows the distribution of MI in controls, in patients with inactive RAS and in patients with active RAS. Strep 2A extracts were used as antigen in these experiments. The MI medians in the samples are illustrated and seen to be 0.97, 0.89 and 0.78 respectively. Statistical analysis (Mann-Whitney U test) shows that MI is significantly smaller in the group of patients with inactive RAS and in the group of patients with active RAS than in the group of controls ($p = 0.0481$, $p < 0.00005$).

Furthermore, MI in the group of patients with active RAS is significantly smaller than MI in the group of patients with inactive RAS ($0.01 < p < 0.05$).

Fig. 2 shows that the distributions are similar if Strep M5 is used as antigen. The MI medians were 0.93, 0.77 and 0.70 respectively. Statistical analysis (Mann-Whitney U test) shows that the differences between controls and patients with inactive or active RAS respectively is equally significant as if Strep 2A is used as antigen ($p = 0.0020$, $p = 0.0001$). The statistical analysis did not reveal any significant difference between the *distribution of MI in* patients with active RAS in relation to the distribution in patients with inactive RAS ($p > 0.10$).

The results of the LMT using AHOM as antigen are shown in Fig. 3. Distributions were found to be similar to those to be observed if Strep 2A or Strep M5 were used as antigens. The MI medians were 0.94, 0.85 and 0.83 respectively. Statistical analysis (Mann-Whitney U test) shows that the MI is significantly smaller in the patients with inactive or active RAS than in the controls ($p = 0.0050$, $p = 0.0128$). Any significant differences in the leucocyte migration in patients with inactive RAS in relation to patients with active RAS was not revealed ($p > 0.10$).

The HMI against *Strep 2A* in controls

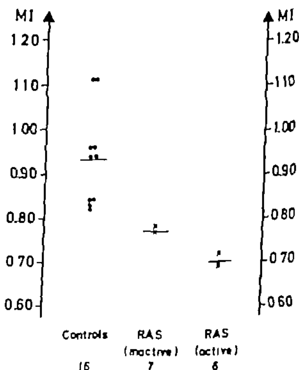


Fig. 2. Leucocyte migration test (LMT) with Strep M5 (2 µg protein per ml) showing the migration indices (MI) of 16 normal controls, 7 patients with inactive recurrent aphthous stomatitis (RAS) and 6 patients with active RAS.

TABLE 1 *The Distribution of Endpoint Titres (EPT) of the Different Immunoglobulins against Strep.2A in 23 Controls and 24 Patients with Recurrent Aphthous Stomatitis (RAS). Sixteen of These Patients Were in a Period with Remission and Eight Were in a Period with Exacerbation of RAS*

Strep. 2A	Immunoglobulin class	Endpoint titres								
		0	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Controls	IgG	1	0	4	10	4	3	1	0	0
N = 23	IgM	12	3	6	2	0	0	0	0	0
	IgA	8	9	4	2	0	0	0	0	0
Recurrent aphthous stomatitis	IgG	0	0	5	0	6	2	6	4	1
N = 24	IgM	11	9	1	1	2	0	0	0	0
	IgA	6	6	7	4	1	0	0	0	0
Recurrent aphthous stomatitis in a period of remission	IgG	0	0	3	0	3	2	4	3	1
N = 16	IgM	7	7	0	1	1	0	0	0	0
	IgA	4	4	4	3	1	0	0	0	0
Recurrent aphthous stomatitis in a period of exacerbation	IgG	0	0	2	0	3	0	2	1	0
N = 8	IgM	4	2	1	0	1	0	0	0	0
	IgA	2	2	3	1	0	0	0	0	0

and in patients with RAS is shown in Table 1. Immunoglobulins against Strep 2A were detected in sera from patients with RAS as well as in sera from controls. The antibodies belong to the immunoglobulin classes IgG, IgM, and IgA. The IgG endpoint titres (EPT) against Strep 2A were significantly higher in the group of patients with RAS than in the controls (Mann-Whitney U test, $p = 0.0043$). The distribution of IgM or IgA endpoint titres (EPT) against Strep. 2A in sera from RAS did not differ significantly from the distributions in the sera from controls ($p > 10$, $p > 10$). Furthermore, the statistical analysis

TABLE 2 *The Distribution of Endpoint Titres (EPT) of the Different Immunoglobulins against Strep.M5 in 16 Controls and 13 Patients with Recurrent Aphthous Stomatitis (RAS). Seven of These Patients Were in a Period with Remission and Six Were in a Period with Exacerbation of RAS*

Strep. M5	Immunoglobulin class	Endpoint titres						
		0	1:1	1:2	1:4	1:8	1:16	1:32
Controls	IgG	3	1	3	6	2	1	0
N = 16	IgM	15	1	0	0	0	0	0
	IgA	15	1	0	0	0	0	0
Recurrent aphthous stomatitis	IgG	0	0	3	3	2	3	2
N = 13	IgM	13	0	0	0	0	0	0
	IgA	12	0	1	0	0	0	0
Recurrent aphthous stomatitis in a period of remission N = 7	IgG	0	0	3	0	1	2	1
Recurrent aphthous stomatitis in a period of exacerbation N = 6	IgG	0	0	0	3	1	1	1

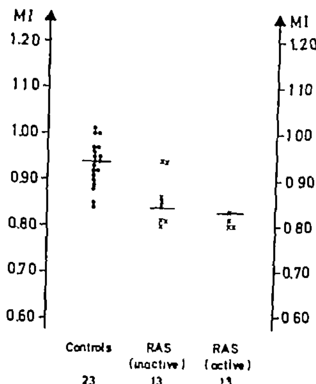


Fig 3 Leucocyte migration test (LMT) with adult human oral mucosa (AHOM) (250 μg protein per ml) showing the migration indices (MI) of 23 normal controls, 13 patients with inactive recurrent aphthous stomatitis (RAS) and 13 patients with active RAS

did not reveal any significant differences in the distribution of any of the immunoglobulins against Strep 2A in patients with active RAS in relation to patients with inactive RAS ($p>0.10$) Sera from one control and one patient with inactive RAS were not available for this part of the IF-study

The HMI against Strep M5 in controls and in patients with RAS is demonstrated in Table 2 Only one control revealed IgM against Strep M5 One control and one patient with RAS revealed IgA against Strep M5 IgG against Strep M5 was detected in sera from RAS as well as from controls Statistical analysis (Mann Whitney U test) of the distribution of IgG endpoint titres (EPT) against Strep M5 in the patients with RAS in relation to the distribution in the conrols shows that EPT against Strep M5 were significantly raised in the patients with RAS ($0.01<p<0.05$) Any significant differences in the distribution of IgG EPT against Strep M5 in patients with active RAS in relation to patients with inactive RAS was not revealed ($p>0.10$)

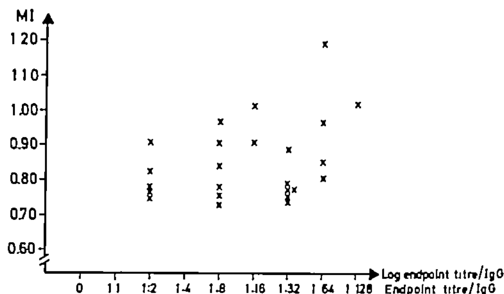
The HMI against AHOM in controls and

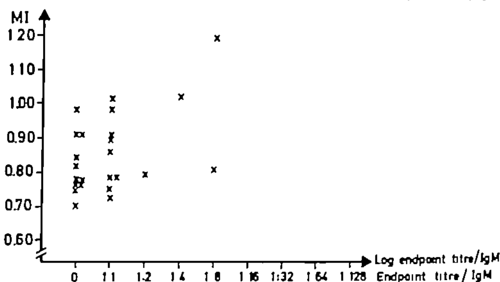
TABLE 3 *The Distribution of Endpoint Titres (EPT) of the Different Immunoglobulins against Adult Human Oral Mucosa (AHOM) in 23 Controls and 24 Patients with Recurrent Aphthous Stomatitis (RAS) Twelve of These Patients Were in a Period with Remission and Twelve Were in a Period with Exacerbation of RAS*

Adult human oral mucosa	Immunoglobulin class	Endpoint titres 0	1 1	1 2	1 4	1 8	1 16	1 32
Controls N = 23	IgG	6	7	6	4	0	0	0
	IgM	0	8	10	4	1	0	0
	IgA	2	11	7	2	1	0	0
Recurrent aphthous stomatitis N = 24	IgG	0	4	11	5	4	0	0
	IgM	0	0	10	6	5	2	1
	IgA	7	9	6	1	1	0	0
Recurrent aphthous stomatitis in a period of remission N = 12	IgG	0	2	5	2	3	0	0
	IgM	0	0	6	2	2	1	1
	IgA	4	5	2	1	0	0	0
Recurrent aphthous stomatitis in a period of exacerbation N = 12	IgG	0	3	5	3	1	0	0
	IgM	0	0	4	4	3	1	0
	IgA	2	5	4	0	1	0	0

TABLE 4. *The Endpoint Titres (EPT) against Strep 2A and Adult Human Oral Mucosa (AHOM) in Sera from 7 Patients with Recurrent Aphthous Stomatitis (RAS). The EPT Were Determined in Sera which Were Taken in Relation to Exacerbation of RAS with Intervals of [illegible] Wk between the Samples*

Patient number	Immuno-globulin class	Antigen	Sample number 1	2	3	4	5
1	IgG	Strep. 2A	1:8	1:4	1:16	1:4	
		AHOM	1:1	1:1	1:2	1:2	
	IgM	Strep. 2A	0	0	1:2	0	
		AHOM	1:2	1:4	1:2	1:4	
	IgA	Strep. 2A	0	0	1:1	0	
		AHOM	1:1	0	0	0	
2	IgG	Strep. 2A	1:8	1:4	1:1	1:4	
		AHOM	1:1	1:2	1:1	1:1	
	IgM	Strep. 2A	0	0	0	0	
		AHOM	1:2	1:2	1:1	1:4	
	IgA	Strep. 2A	1:1	1:1	0	1:1	
		AHOM	0	0	0	0	
3	IgG	Strep. 2A	1:32	1:32	1:32		
		AHOM	1:4	1:2	1:4		
	IgM	Strep. 2A	0	1:1	0		
		AHOM	1:2	1:8	1:4		
	IgA	Strep. 2A	0	1:1	0		
		AHOM	1:1	0	1:1		
4	IgG	Strep. 2A	1:32	1:32	1:32	1:32	
		AHOM	1:4	1:8	1:4	1:8	
	IgM	Strep. 2A	0	0	0	0	
		AHOM	1:2	1:2	1:8	1:2	
	IgA	Strep. 2A	1:2	1:1	1:1	1:2	
		AHOM	1:1	1:1	1:4	1:1	
5	IgG	Strep. 2A	1:64	1:32	1:16		
		AHOM	1:16	1:8	1:8		
	IgM	Strep. 2A	1:4	1:2	1:4		
		AHOM	1:8	1:8	1:32		
	IgA	Strep. 2A	0	0	0		
		AHOM	0	0	0		
6	IgG	Strep. 2A	1:4	1:2	1:8	1:4	
		AHOM	1:4	1:2	1:2	1:2	
	IgM	Strep. 2A	1:1	0	0	0	
		AHOM	1:1	1:2	1:2	1:2	
	IgA	Strep. 2A	0	0	1:1	0	
		AHOM	1:1	0	1:1	0	
7	IgG	Strep. 2A	1:32	1:8	1:4	1:4	1:2
		AHOM	1:2	1:2	1:1	0	1:1
	IgM	Strep. 2A	1:8	1:2	1:1	1:1	0
		AHOM	1:4	1:1	1:4	1:4	1:4
	IgA	Strep. 2A	1:1	1:2	1:2	1:1	0
		AHOM	1:2	0	1:1	0	0





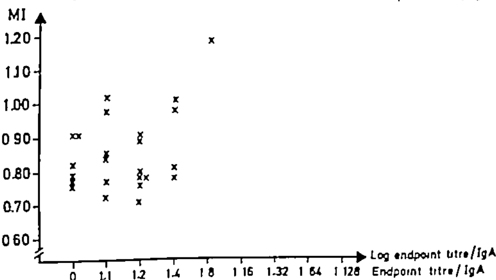


Fig 4 The relationship between antibodies (IgG IgM IgA) and migration indices (MI) against Strep. 2A in 24 patients with recurrent aphthous stomatitis (RAS)

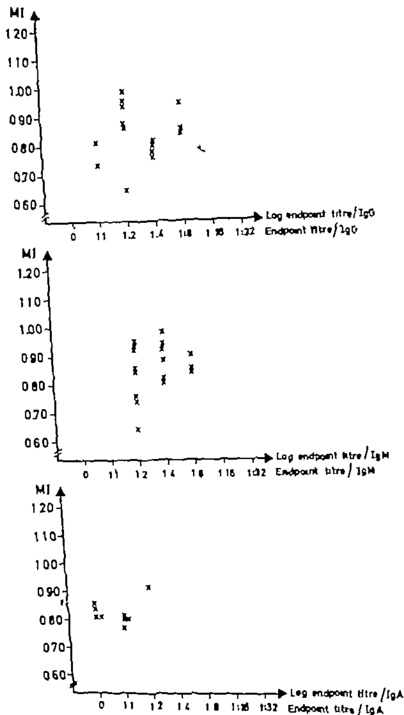


Fig. 5. The relationship between antibodies (IgG, IgM, IgA) and migration indices (MI) against adult human oral mucosa (AHOM) in 24 patients with recurrent aphthous stomatitis (RAS)

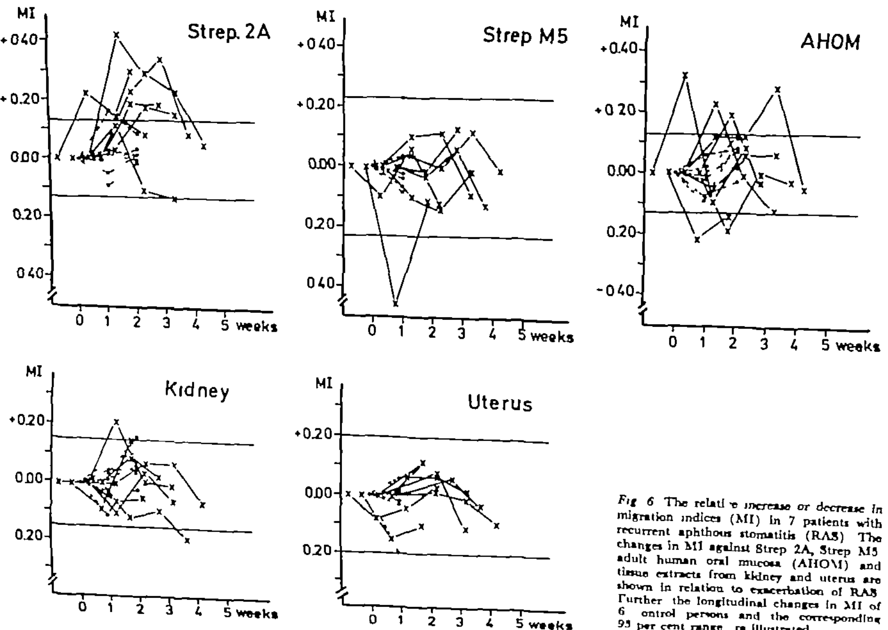


Fig 6 The relati e *increase or decrease in* migration indices (MI) in 7 patients with recurrent aphthous stomatitis (RAS) The changes in MI against Strep 2A, Strep M5 adult human oral mucosa (AHOM) and tissue extracts from kidney and uterus are shown in relation to exacerbation of RAS Further the longitudinal changes in MI of 6 ontrol persons and the corresponding 95 per cent range re illustrated

in RAS is presented in Table 3. Immunoglobulins against AHOM were detected in sera from RAS as well as in sera from controls. The detected antibodies against AHOM belong to the immunoglobulin classes IgG IgM, and IgA. The IgG and IgM endpoint titres against AHOM were significantly higher in the groups of patients with RAS than in the controls (Mann-Whitney U-test, $p = 0.0046$, $p = 0.0004$) The distribution of IgA EPT against AHOM in RAS did not differ significantly from the distribution in the controls ($p > 0.10$) No significant differences in the distributions of any of the immunoglobulins against AHOM were demonstrable by a comparison of sera from patients with active RAS and sera from patients with inactive RAS ($p > 0.10$) Sera from one inactive and one active patient with RAS were not available for this part of the IF-study

The detected antibodies against Strep 2A, Strep. M5 and AHOM were able to bind serum complement as similar immunofluorescence patterns were revealed if a rabbit anti C3 fluorescein isothiocyanate (FITC) conjugate (Dakopatt's code No. F 1062) was used instead of the rabbit anti IgG IgM, and IgA conjugates, respectively The anti C3 immunofluorescence reactions were difficult to read when the sera were titrated. However the EPT to be detected by the different antigens seemed to be in agreement with the EPT of IgG and/or IgM.

Figs. 4 and 5 show the relationships between EPT and MI against Strep. 2A and AHOM respectively in patients with RAS. There is apparently no correlation between any of the immunoglobulins IgG IgM, and IgA and the corresponding MI Furthermore the present investigation did not reveal any correlation between IgG endpoint titre and MI against Strep. M5 in RAS

Fig 6 shows the results obtained in the longitudinal part of the investigations of the CMI against Strep 2A, Strep M5 AHOM, kidney and uterus in 7 patients with RAS and in 6 controls. Six of the patients showed a significant, relative increase in MI in relation to exacerbation when Strep. 2A was used as antigen. One control showed a similar increase in the longitudinal experiments. If AHOM was used as antigen, four patients with RAS showed a significant, relative increase in MI and two patients revealed a significant, relative decrease One patient with RAS was neither stimulated nor inhibited in relation to exacerbation. The CMI against Strep. M5 was significantly decreased in one patient with RAS in relation to exacerbation. One patient showed a significant increase and one showed a significant decrease in CMI against the kidney extract. None of the patients with RAS or the controls revealed any significant increase or decrease if the extract from the uterus was used as antigen.

The limits for significant, relative increase or decrease in MI were calculated on the basis of the longitudinal observations in the six controls. The limits were calculated as the mean change in MI to which two standard deviations were added. Statistical analysis (Hypergeometric distribution) of the results illustrated in Fig 6 shows that the changes in MI against Strep. 2A and AHOM in RAS differ significantly from the changes in MI in the controls ($0.01 < p < 0.05$ $p < 0.01$) The relative increase or decrease of MI in RAS against Strep. M5 kidney and uterus did not differ significantly from the changes of MI in the controls ($p > 0.10$)

The changes in EPT against Strep. 2A and AHOM in relation to exacerbation of RAS in seven patients are seen in Table 4 No systematical changes were detected in any of the immunoglobulins against Strep. 2A and AHOM in relation to the exacerbation of RAS

DISCUSSION

Recently LMT experiments have revealed a significantly increased *in vitro* CMI against Strep. 2A, Strep. M5 and AHOM in patients with RAS (5) The present investigation shows that the above CMI is significantly increased in patients with inactive RAS as well as in those with active RAS.

The CMI against Strep. 2A was signifi-

cantly changed in relation to exacerbation of RAS both in the cross-sectional part and in the longitudinal part of the present study. The Strep. 2A suspension used in the longitudinal LMT experiments seems to be a weaker antigen than the sonicated Strep 2A extract (5). The relative leucocyte migration stimulation instead of migration inhibition shown in Fig. 6 might be caused by such a weak antigen. Therefore, the present LMT results are consistent with an increased CMI against Strep 2A in RAS in relation to exacerbation. Furthermore, this *in vitro* result is consistent with the finding that the positive skin reaction against different streptococcal antigen suspensions is raised parallel with the frequency of recurrence of RAS (17).

The CMI against Strep M5 was increased in the group of patients with inactive RAS and the group of patients with active RAS. However, the CMI against Strep M5 was not significantly changed in the group of patients with active RAS in relation to those with inactive RAS, and it was not either significantly changed in relation to the exacerbation of RAS in the longitudinal LMT investigation. These findings might indicate that Strep 2A and Strep M5 share common antigen determinants, but that Strep 2A possesses antigen determinants which are more specifically correlated with RAS. The AHOM extract used failed to reveal any significant difference between the groups of patients with active and inactive RAS. Nevertheless, a significant change in CMI against AHOM was demonstrated in relation to the exacerbation of RAS. A high, non-specific toxicity of the AHOM tissue extract which prevents the use of high antigenic concentrations in the migration cultures, and a varying sensitivity of the seven patients with RAS (Fig. 6) might explain the LMT results obtained. The correlation between clinical exacerbation of RAS and the leucocyte migration response against AHOM is apparently not equally clear-cut as the correlation between the lymphocyte transformation and the lymphocyte cytotoxicity responses (15, 16). The explanation of this discrepancy may be that different antigens were used and different groups of patients were studied. Another explanation may be that the production of blastogenic and cytotoxic lymphokines is more intense than the production of MIF when lymphocytes from active RAS interact with oral mucosa.

The HMI against Strep 2A, Strep. M5 and AHOM was significantly raised in the different groups of patients with RAS presented here. This is in agreement with previous investigations (4, 7, 8, 11, 12). In previous IF-studies of the HMI against Strep. 2A and AHOM a mixture of rabbit anti IgG and IgM conjugate was used (7, 8). The present investigation shows that the streptococcal antibodies which were significantly raised in RAS belong to the immunoglobulin class IgG. A high percentage of streptococci belonging to groups A, C, and G was found to react with IgG in a protein A like, non-immune way (10). As Strep M5 belongs to *Streptococcus pyogenes* group A, the fluorescence to be detected if Strep M5 was used as antigen might be caused by such non-immune, protein A like reactivity. From a statistical point of view, the above unspecific protein A like reaction is expected to be uniformly distributed in sera from controls and RAS respectively. The demonstration of significantly raised EPT for the fluorescence reactions in the sera from RAS indicate that an antigen-antibody reaction with the highest EPT in the sera from RAS also is involved. The Strep 2A belongs to *Streptococcus sanguis* group H which, to the knowledge of the present author, does not possess protein A-like characteristics.

The auto-antibodies involved belong to the immunoglobulin classes IgG and IgM. This characterization of the auto-antibodies in RAS is in agreement with a previous characterization of the auto-antibodies against oral mucosa (14, 15). As in the study of agglutinating auto-antibodies in RAS (15), the present IF study did not reveal any significant changes in the auto-antibodies against AHOM in relation to exacerbation. Furthermore, the IF-experiments did not reveal any significant

changes in the EPT against streptococcal antigens in relation to activity of RAS. Any correlation between EPT and MI against any of the antigens used in the present study was not revealed. As far as the oral mucosa is concerned, the result is in support of the findings obtained by agglutination test and lymphocyte transformation test (15). The present and previous studies indicate that CMI against streptococcal and oral mucosa antigens might be involved in the pathogenesis of RAS (2, 3, 5, 6, 13, 15, 16). Suggestions concerning the role of the HMI against streptococcal and oral mucosa antigens in the pathogenesis of RAS seem more uncertain. Antibodies against the above antigens have been demonstrated in several studies (1, 4, 7, 8, 11, 12, 14, 15). Furthermore, the present study shows that the antibodies against streptococcal and oral mucosa antigens in RAS belong to the immunoglobulin classes IgG and IgM and that they are able to bind C3. Accordingly humoral mediated immunity (cytotoxic type of hypersensitivity type II and/or complex mediated hypersensitivity type III) and/or cell-mediated immunity (cell-mediated hypersensitivity type IV) may be involved in the immunopathogenesis of RAS. In conclusion, the present findings are in support of those obtained in previous studies according to which CMI and HMI against streptococcal antigens (*Streptococcus sanguis* strain 2A and *Streptococcus pyogenes* strain M5) and oral mucosa antigens are characteristic features of RAS and indicate that the CMI against *Strep.* 2A and adult human oral mucosa (AHOM) is significantly increased in relation to exacerbation of RAS.

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REFERENCES

1. *Brody H. A. & Silverman S.* Studies on recurrent oral aphthae I. Clinical and laboratory comparisons. Oral Surg. 27: 27–34, 1969.
2. *Dolby A. E.* Recurrent aphthous ulceration. Effect of sera and peripheral blood lymphocytes upon oral epithelial tissue culture cells. Immunology 17: 709–714, 1969.
3. *Dolby A. E.* Mikulicz recurrent oral aphthae. The effect of anti-lymphocyte serum upon the in vitro cytotoxicity of lymphocytes from patients for oral epithelial cells. Clin. Exp. Immunol. 7: 681–686, 1970.
4. *Donatsky O.* An immunofluorescence study on the cross-reaction between Strep. 2A and human oral mucosa. Scand. J. Dent. Res. 83: 111–119, 1975.
5. *Donatsky O.* A leucocyte migration study on the cell-mediated immunity against adult human oral mucosa and streptococcal antigens in patients with recurrent aphthous stomatitis. Acta path. microbiol. scand. Sect. C 84: 227–234, 1976.
6. *Donatsky O. & Bendixen, G.* In vitro demonstration of cellular hypersensitivity to Strep. 2A in recurrent aphthous stomatitis by means of the leucocyte migration test. Acta allergol. 27: 137–144, 1972.
7. *Donatsky O. & Dabelsteen E.* An immunofluorescence study on the humoral immunity to Strep. 2A in recurrent aphthous stomatitis. Acta path. microbiol. scand. Sect. B, 82: 107–112, 1974.
8. *Donatsky O. & Dabelsteen, E.* An immunofluorescence study on the humoral immunity to adult human oral mucosa in recurrent aphthous stomatitis. Acta allergol. 29: 308–318, 1974.
9. *Graykowski E. A., Barile M. F., Lee W. B. & Stanley H. R.* Recurrent aphthous stomatitis. Clinical, therapeutic, histopathologic and hypersensitivity aspects. J. Amer. med. Ass. 196: 637–644, 1966.
10. *Kronvall G.* A surface component in group A, C and G streptococci with non immune reactivity for immunoglobulin G. J. Immunol. 111: 1401–1406, 1973.
11. *Lehner T.* Recurrent aphthous ulceration and autoimmunity. Lancet ii: 1154–1155, 1964.
12. *Lehner T.* Autoimmunity and management of recurrent oral ulceration. Br. dent. J. 122: 15–20, 1967.
13. *Lehner T.* Stimulation of lymphocyte transformation by tissue homogenates in recurrent oral ulceration. Immunology 13: 159–166, 1967.
14. *Lehner T.* Pathology of recurrent oral ulceration and ulceration in Behcet's syndrome. Light, electron and fluorescence microscopy. J. Path. 97: 481–494, 1969.
15. *Lehner T.* Characterization of mucosal antibodies in recurrent aphthous ulceration and

Behcet s syndrome Arch. oral Biol. *14* 843–853 1969

16 *Rogers R. S Sams W M & Shorter R G* Lymphocytotoxicity in recurrent aphthous stomatitis Lymphocytotoxicity for oral epithelial cells in recurrent aphthous stomatitis and Behcet s syndrome Arch Derm. *109* 361–363 1974

17 *Sallay K., Hajós M K & Bánóczy J.* Untersuchungen über den allergischen Pathomechanismus der chronischrezidivierende Aphthen. Allergie u Immunologie *17* 17–23 1971

18. *Shore R N & Shelley W B.* Treatment of aphthous stomatitis by suppression of intralesional streptococci. Arch. Derm. *109* 400–402, 1974

Acta path. microbiol. scand. Sect. C, 84 283–289 1976

TRANSPLANTATION OF HUMAN ADIPOSE TISSUE TO NUDE MICE

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Bach-Mortensen, N Remert, P & Ballegaard, S. Transplantation of human adipose tissue to nude mice. Acta path. microbiol scand. Sect. C, 84 283–289 1976.

Human adipose tissue was transplanted to the mouse mutant nude (nu/nu) All the grafts were accepted and contained fat cells easily distinguishable from those of the mouse. No detectable relation between the histological pictures before and after grafting was found. In some transplants nerve tissue, and in others macrophages containing fat droplets, were found. The fat tissue graft might be useful for investigation of the influence of various hormones on human fat cells.

Key words Nude mouse transplantation human adipose tissue.

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The mouse mutant nude (nu/nu) accepts different kinds of grafts, which may be explained by its lack of a thymus (*Pantelouris* 1968) First it was shown that the nude mouse did not reject heterografts such as rat skin, human skin and malignant tumors (*Rygaard* 1969 *Rygaard & Povlsen* 1969) and later the same was shown for allografts (*Pennycuik* 1971)

Experimentally it might be possible to investigate the influence of various hormones on human target cells by transplanting these to nude mice and treating the mice. Human testicular and ovarian tissue has already been successfully transplanted to nude mice (*Skakkebæk et al.* 1974) Human fat cells are highly responsive to hormonal stimuli, and it would be of interest to be able to examine their reactions to such stimuli in grafts. To investigate whether human fat cells survive after transplantation to nude mice, the following experiments were carried out.

MATERIALS AND METHODS

Recipient Mice

Nude mice of BALB/c background (4–5 back cross in gene transfer) were supplied by the Pathological Anatomical Institute, Kommunehospitalet, Copenhagen, and were kept there after the transplantations had been performed. Both male and female mice were used. Three series of adipose tissue grafts were made with 8 to 15 animals in each series.

Human Donor Material

The adipose tissue was obtained from three boys aged 3 12 and 14 years during surgery. None of the patients was severely ill or suffered from any endocrine disorder. At the beginning of the operation, pieces of subcutaneous adipose tissue were taken just proximal to the inguinal ligament. The samples were cut into smaller pieces, approximately 30 cubic millimetres large. Some pieces were

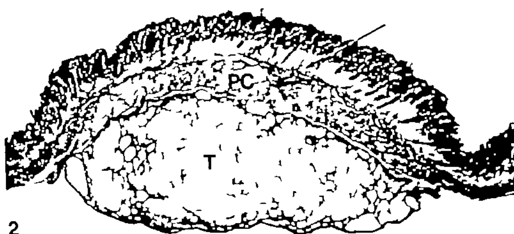
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17 *Sallay K Hajós M K & Bándery J* Untersuchungen über den allergischen Pathomechanismus der chronischrezidivierende Aphthen Allergie u. Immunologie *17* 17–23 1971

18 *Shore R N & Shelley W B* Treatment of aphthous stomatitis by suppression of intralesional streptococci Arch. Derm. *109* 400–402 1974


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used for transplantation and others were immediately fixed. The fixed fat tissue pieces were later dehydrated and embedded in paraffin or Epon 812.

Grafting Technique

Under sterile conditions a piece of adipose tissue was placed in the loose connective tissue layer between the panniculus carnosus and the deep muscular fascia of the mouse. This was done under anaesthesia, through a small incision in the skin of the left side of the back. The location of the graft is seen in Fig. 1. Propanidid (Epontol®) was used as anaesthetic in a dose of 0.5 mg per gram mouse. In each series some mice were killed after 2–4 weeks, and the remainder after 6–8 weeks.

Autopsy and Preparation of Tissue

The grafts were excised immediately and cut into small blocks. Some of the blocks were immersed in a fixative containing 2 per cent formaldehyde and 1.25 per cent glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) for 2 hours, and post fixed in 2 per cent osmium tetroxide with the same buffer for 6–12 hours. After fixation the blocks were dehydrated through increasing concentrations of ethanol and embedded in Epon 812. 1 µm thick sections were cut and stained with toluidine blue. To have a general outline of the graft, a few transplants with surrounding tissue were fixed as a whole and embedded in paraffin. 10 µm thick sections were made and stained using the Feulgen reaction.

RESULTS

Macroscopic Findings

The grafts were seen as slowly growing tumours under the skin (Fig. 1) and they were accepted in all the animals examined. At autopsy the grafts were found fixed on the deep side of the panniculus carnosus, having a thin connective tissue capsule. They were discoid in shape, and approximately 6×4×3 millimetres in size both after 3 weeks and after 8 weeks. Large vessels were seen connecting the grafts with the surrounding tissue.

Microscopic Findings

A cross-section of the whole graft showed that the fat cells in the graft were distinctly separated from the mouse fat cells of the subcutaneous layer by the panniculus carnosus (Fig. 2). The mouse fat cells were also smaller than the fat cells in the grafts. The graft was enclosed by a thin capsule containing many collagenous fibres and fibroblasts. Trabeculae consisting of connective tissue arose from the capsule and penetrated the graft for varying distances (Fig. 2). In the connective tissue of the graft many vessels (mostly arterioles and venules) and some large and small nerves were seen (Fig. 3). In the fat tissue *fixed immediately after excision from the* donors, nerve tissue was not seen.

Most of the graft was composed of fat cells (Fig. 2), the morphology of which varied from graft to graft. Some grafts contained either unilocular or multilocular fat cells, while others contained both types. The unilocular cells were large, from 60 to 120 µm *in diameter*, had *a spherical or polyhedral* shape, and contained a single droplet of fat, reducing the cytoplasm to a thin rim (Fig. 4). The multilocular cells were smaller, from 30 to 60 µm in diameter, had a spherical or ovoid shape, and contained large and small droplets of fat. The cytoplasm was more abundant and contained many mitochondria (Fig. 5).

In the two to four week old grafts fat engulfing cells, probably macrophages, were found in varying numbers. These cells were located especially at the periphery of the graft in close relation to large unilocular fat cells. Typically they contained a large number of spherical fat droplets. They were easily *distinguished from multilocular* fat cells, as the fat droplets in the macrophages were

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Fig. 1. The photograph shows a nude mouse under light anaesthesia. This mouse has carried a transplant for 22 days. The arrow points to the edge of the graft.

Fig. 2. Photomicrograph of a paraffin section through the skin and transplant 30 days after grafting. The transplant (T) is seen deep to the *panniculus carnosus* (PC). *The arrow* points to the subcutaneous fat tissue of the mouse. × 25

Fig. 3. Photomicrograph of a paraffin section *through part of a 30 days old transplant.* Note the many blood vessel (V) and a large nerve (N). F. Fat cell. PC. Panniculus carnosus. Arrow indicates the graft capsule. × 200

TABLE 1 *Relation with Respect to Fat Cell Size and Type between Donor Tissue and Corresponding Graft after 2 to 8 Weeks*

Donors	Size and type of fat cells	
	In donor tissue	In graft tissue
Boy 12 years	30–50 μm Many multilocular	c. 100 μm unilocular and c. 50 μm multilocular
Boy 14 years	100–150 μm Unilocular	100–150 μm unilocular and a few c. 50 μm multilocular
Boy 3 years	70–100 μm Unilocular	50–100 μm unilocular and 50 μm multilocular

small, of uniform size, and lightly stained (Fig. 6) Monocytes were often found in blood vessels of the graft, typically adherent to the endothelium. Many capillaries were found between the fat cells in all of the grafts (Figs. 5 and 6)

In each series of experiments comparisons were made with respect to fat cell size and type before and after grafting. No detectable relations between the histological pictures before and after grafting were found in these experiments. The results of the comparisons are outlined in Table 1 No difference in the morphology of the transplants could be demonstrated between male and female mice. No variations in the morphology of the fat cells in the adipose tissue of the nude mice examined were found. Most subcutaneous fat cells were unilocular and 20 to 40 μm in size.

Fig 4 Photomicrograph of 1 μm thick section through 22 days old transplant. Most of the fat cell are unilocular × 600.

Fig 5 Photomicrograph of a 1 μm thick section through 22 days old transplant Most of the fat cells are multilocular Arrows indicate mitochondria. × 600

Fig 6 Photomicrograph of 1 μm thick section through the peripheral part of 22 days old transplant Note the many fat-engulfing cells, probabl macrophages (M) containing many small lightly stained lipid droplets of equal size F Fat cell × 1500

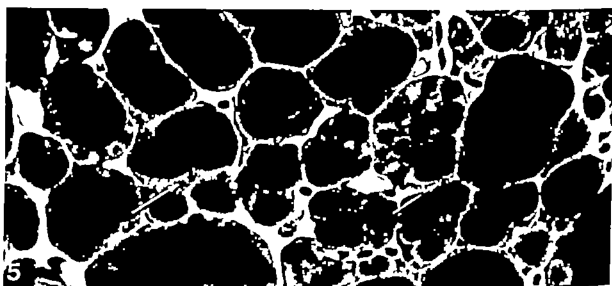
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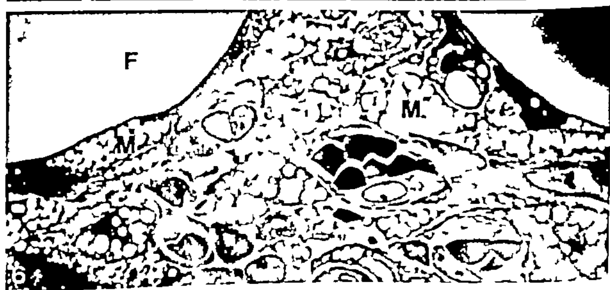
Our experiments have shown that human adipose tissue survives when transplanted to the nude mouse. The grafts were well supplied with vessels, and rather large nerves grew from the mouse into the graft. The grafts increased in size, mostly during the first and second week after transplantation. No animals were killed during that period, and further experiments have to be performed to elucidate the cytology during this interval. No mitoses were seen in the grafts from animals killed 2 to 8 weeks after transplantation. The number of fat cells in the transplants before and after grafting has not been determined, but since the grafts grew cell divisions of pre-adipocytes or other precursors of fat cells cannot be excluded. The amount of connective tissue in the grafts was small.

All the multilocular fat cells looked vital with much cytoplasm and many mitochondria. Some of the unilocular fat cells in the periphery of the younger grafts were probably necrotic and losing fat, which was engulfed by macrophages. These fat cells may have been damaged during the excision of the adipose tissue from the donors, or during the cutting of the transplants, since the unilocular cells in the center of the graft appeared vital.

As regards the analysis of the fat cell morphology we realize that present knowledge of adipose tissue histogenesis and histo-


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Prenycalk P R.: Unresponsiveness of nude mice to skin allografts. Transplantation *11* 417–418, 1971

Poznanski, W J., Waheed, I & Van R., Human fat cell precursors. Morphologic and metabolic differentiation in culture Lab. Invest. *29* 570–576, 1973

Rygaard, J Immunobiology of the mouse mutant "nude" Preliminary investigations. Acta path. microbiol. scand. *77* 761–762, 1969

Rygaard J & Povlsen C O Heterotransplantation of a human malignant tumour to "nude" mice. Acta path. microbiol. scand *77* 758–760 1969

Skakkebaek, N E., Jensen, G Povlsen, C O & Rygaard, J Heterotransplantation of human foetal testicular and ovarian tissue to the mouse mutant nude. Acta Obstet. Gynec. scand. *53* 73–75 1974

Slavin B O & Elias J J The influence of pituitary hormones and norepinephrine on the size of adipose cells in organ culture. Anat. Rec. *164* 141–152, 1969

Slavin B. G The cytophysiology of mammalian adipose cells. Int. Rev Cytol. *33* 297–334 1972.

Smith, U., Morphologic studies of human subcutaneous adipose tissue in vitro. Anat. Rec. *169* 97–104 1970.

Tanuma, Y Yamamoto M Ito T & Yokochi C., The occurrence of brown adipose tissue in perirenal fat in Japanese. Arch. hist. jap. *38* 43–70 1975

Vodovar N., Desnoyers F & Flanzy J.: Depot adipeux mesenterique d porcelet. Étude morphologique. Ann biol. anim. bioch. biophys. *12* 243–262, 1972.

physiology is too limited to permit interpretation of our findings. The histogenesis of adipose tissue is still under debate (*Bloom & Fawcett* 1975) and many problems concerning distribution and significance of multilocular fat cells remain to be solved even if much information has been obtained (*Johansson* 1959, *Aherne & Hull* 1966, *Feyrter* 1973, *Tanuma et al* 1975). In children, differences in subcutaneous fat cell size according to the site of sampling have been reported (*Duckerts & Bonnet* 1973). Number and size of fat cells have been reported to vary in normal and obese children (*Bonnet et al* 1970). Most published studies concerning the process of fat accretion during development of obesity in man suggest that the adipose tissue mass grows principally by increase in size rather than in number of fat cells (in adults) while others conclude that most stroma cells of human adipose tissue are differentiated precursors (pre-adipocytes) with a potential for multiplication and for evolving into mature fat cell types (*Poznanski et al* 1973). A main reason for the limited knowledge of fat cell morphology is the technical problems invariably encountered during fixation, embedding and cutting of adipose tissue (*Napolitano* 1965, *Vodovar et al* 1972).

Many attempts have been made to describe the cytological changes occurring in human adipose cells undergoing lipid mobilization or deposition and to correlate these changes with concomitant functional activities. But a prevailing lack of sufficient information has made it difficult to present a concise analysis of the subject (*Slavin* 1972). Many experiments concerning hormonal influences on fat cell morphology, physiology and biochemistry have been performed on free fat cells (*Jeanrenaud* 1970), and on organ cultures of adipose tissue (*Slavin & Elias* 1969, *Smith* 1970). Hormonal influence on free fat cells is not necessarily identical with the hormonal influence on intact tissue, or with the *in vivo* effect of the hormone: human adipose tissue segments are more sensitive to insulin than isolated fat cells, even when both derive from the same donor (*Gries & Steinke* 1967). During the preparation of the isolated fat cells, hormonal receptors may be disturbed.

We think that our nude mouse/human adipose tissue graft model might be a suitable tool for examining the influence of various human hormones on the fat cell, as this model imitates the physiological situation better than isolated fat cells or tissue segments.

The authors wish to thank *Margit Bækted* and *Oluf Rasmussen* (Pathological Anatomical Institute, Kommunehospitalet, Copenhagen), *Susan Max Jacobsen* and *Kjeld Stub* (Anatomy Department C, University of Copenhagen) for skilled technical assistance. We are grateful to Professors *H. Moe*, *H. Andersen*, *J. Rygaard* and *K. Mennsen* for help and advice.

REFERENCES

Aherne W. & Hull D.: Brown adipose tissue and heat production in the newborn infant. J. Path. Bact. *91*: 223–234, 1966.

Bloom W. & Fawcett D. W.: A textbook of histology. 10th ed. W. B. Saunders, Philadelphia & London 1975, p. 196–208.

Bonnet F., Gosselin L., Chantraine J. & Senterre J.: Adipose cell number and size in normal and obese children. Rev. Europ. Études Clin. et Biol. *15*: 1101–1104, 1970.

Duckerts M. & Bonnet F.: Adipose cell size differences related to the site of adipose tissue biopsy in children. Biomedicine *19*: 214–216, 1973.

Feyrter F.: Ein adrenolipoides Syndrom. Normologie und Pathologie des braunen Fettgewebes des Menschen. Georg Thieme Verlag, Stuttgart 1973, p. 1–59.

Gries F. A. & Steinke J.: Comparative effects of insulin on adipose tissue segments and isolated fat cells of rat and man. J. Clin. Invest. *46*: 1413–1421, 1967.

Jeanrenaud B., Hepp D. & Renold A. E.: The influence of lipolysis on energy metabolism of isolated fat cells. In: Jeanrenaud B. & Hepp D. (Eds.): Adipose Tissue Regulation and metabolic functions. Georg Thieme Verlag, Stuttgart & Academic Press, New York and London 1970, p. 76–79.

Johansson B.: Brown fat: a review. Metabolism *8*: 221–240, 1959.

Napolitano L. M.: Observations on the fine structure of adipose cells. Ann. N.Y. Acad. Sci. *131*: 34–42, 1965.

Pantelouris E. M.: Absence of thymus in a mouse mutant. Nature (London) *217*: 370–371, 1968.

MATERIAL AND METHODS

The series comprises 52 children, four to fifteen years of age, who were tonsillectomized at the Otological Department, Aalborg Hospital, North. Data on the children are published elsewhere (*Østergaard* 1975). The 52 patients were divided into two groups according to age and sex as shown in Table 1. The distribution of males and females was equal in the younger age group (four to nine years of age), whereas females predominated in the older age group (ten to fifteen years of age). Except for frequently occurring tonsillitis and, in some of the patients, a tendency to atopic diseases, the children were otherwise healthy. Thirty-one of the patients came from the town itself, the others from the nearby rural districts. Nearly all socio-economic groups were represented.

Three to six months (mean four months) after tonsillectomy all the patients were seen again for medical check-up and collection of samples. On this occasion they were especially asked about the frequency of infections including sore throats in the intervening time.

Controls. Fifty-two healthy controls were selected according to age and sex to match the patients. None of the controls had experienced recurrent infections including sore throats. All of the controls (or their parents) were individually interviewed by a medical practitioner concerning health, and the throat was inspected to safeguard that the tonsils were in situ and offered no signs of chronic infection. None of the controls had experienced an earlier adenoidectomy and most of them came from the same geographic area and socio-economic groups as the patients. When they were seen, serum and saliva were collected and frozen immediately at -20° C until determination of immunoglobulins.

Serum and saliva samples were collected from all the patients before and three to six months (mean four months) after tonsillectomy. Saliva was sucked directly into a test tube from the floor of the mouth without stimulation and at least one hour after a meal. The samples were frozen immediately and stored at -20° C. After the saliva had been thawed, mucous debris had sedimented in most of the samples leaving a clear supernatant for immunoglobulin determination only. A few saliva samples needed light centrifugation. The saliva was always tested with haemastix for blood contamination and found negative. None of the patients offered signs of inflammatory processes of the mouth.

Serum and saliva immunoglobulin assays were performed by the Department of Clinical Chemistry, Aalborg Hospital, South. Rabbit-anti-human IgG, IgA and IgM antisera, obtained from Dacopatts, Copenhagen, were used. Serum immunoglobulins of the classes IgG, IgA and IgM were quantitated by the electro-immuno-technique after carbamylation of the sera (*Weeke* 1968 a, *Weeke* 1968 b). This method was chosen because of its great sensitivity and accuracy (*Laurell* 1972). Pooled human serum, calibrated in terms of the WHO reference preparation, was used as a standard.

IgG, IgA and IgM was measured similarly in the saliva using the same serum standard. When the saliva samples were examined, the serum standard was diluted prior to carbamylation because of the expected, much lower concentration of immunoglobulins in saliva (IgG 0.09–0.80 IU/ml, IgA 0.54–3.68 IU/ml, IgM 2.04–10.31 IU/ml). The sensitivity of detection of saliva immunoglobulins was 0.09, 0.54 and 2.04 for IgG, IgA and IgM, respectively. A control serum was included in every run. The day to day precision (n = 20 days) of the runs was 7.1 per cent, 5.6 per cent and 5.4 per cent for IgG, IgA and IgM, respectively.

Serum IgE estimations were performed by the Department of Clinical Chemistry, Odense Hospital, and were carried out in all the patients before and after tonsillectomy and in all the controls. A radio-immunosorbent test (Phadebas kit, Pharmacia, Copenhagen) described by *Johansson et al.* (1968) was used. The sensitivity of detection of IgE in serum was 10 IU/ml.

Bacterial cultures were performed by the Department of Clinical Microbiology, Aalborg Hospital. A small piece of the tonsil, the size of 1 × 1 mm, was minced with sterile scissors and spread onto blood agar and chocolate agar plates. Incubation was at 35° C aerobically and anaerobically and the plates were read after 24 and 48 hours.

Colonies showing beta-haemolysis and shown to produce soluble haemolysin were registered as beta-haemolytic streptococci and sent to the Streptococcal Department, Statens Seruminstitut, Copenhagen, for group- and type identification. In case of doubt, the colonies would be Gram stained in order to exclude Gram-positive or Gram-negative rods. A CAMP test was made to characterize strains belonging to Lancefields group B.

Non-haemolytic colonies of small Gram-negative rods growing with characteristic grey colonies on chocolate agar and in symbiosis with staphylococci on blood agar were registered as *Haemophilus influenzae*. All strains of *H. influenzae* which exhibited iridescence on Levinthal agar were tested for capsular swelling in a *H. influenzae* type b serum.

Gram-positive cocci in clusters growing with round, smooth, and glistening colonies were registered as *Staphylococcus aureus* if they were able to clot citrated 5 per cent horse plasma.

If mixed flora did not grow with any of the above mentioned potential pathogens it was registered as normal bacterial flora.

Antistreptolysin-O (ASO) antibody determinations were performed by the Streptococcal Department, Statens Seruminstitut, Copenhagen, by the method of *Todd* (1932) modified by *Kalbak* (1942). ASO-titres were determined on patient

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IgA LEVELS AND CARRIER RATE OF *HAEMOPHILUS INFLUENZAE* AND BETA-HAEMOLYTIC STREPTOCOCCI IN CHILDREN UNDERGOING TONSILLECTOMY

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Østergaard, P Aabel IgA levels and carrier rate of *Haemophilus influenzae* and beta haemolytic streptococci in children undergoing tonsillectomy Acta path microbiol. scand Sect. C, *84* 290–298, *1976*.

Studies of IgG IgA, IgM and IgE in serum and of IgG IgA and IgM in saliva were performed in 52 children undergoing tonsillectomy The results revealed that levels of IgA in serum and saliva in the patients were significantly reduced as compared with levels in age- and sex related healthy controls ($p<0.001$ and <0.025 repectively) Recovery of beta-haemolytic streptococci and *Haemophilus influenzae* from the removed tonsils was also well correlated with low IgA in serum ($p<0.01$) A considerable lack of IgA fluorescing plasma cells in tonsillar tissue demonstrated in an earlier study of the same patients was consistent with carriage of beta-haemolytic streptococci and *Haemophilus influenzae* ($p<0.01$) The significant decrease in serum and saliva IgA was only found among the youngest patients in this study The hypothesis is raised that the decreased level of saliva IgA influences the increased tendency at pathogenic bacteria to adhere to and colonize on the tonsil mucosa, and furthermore, the lack of IgA plasma cells in the tonsils supports the view that IgA prevents penetratation of microorganisms through the epithelial surface secondarily establishing an acute inflammation of the tonsils.

Key words IgA levels *Haemophilus influenzae* beta-haemolytic streptococci tonsillectomy

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Much controversial literature concerning the role of the human tonsils in the local and general immune defence has appeared. Clinical and immunological research has been rather scanty and one aim of the present study was therefore to investigate the carrier rate of presumably pathogenic bacteria in children undergoing tonsillectomy and to examine whether carriage might be connected with a decrease in or absence of immunoglobulins in serum and/or saliva Another aim was to compare the results of the immunoglobulin determinations and the carrier rate of pathogenic bacteria in the tonsils with the results obtained in an earlier immunofluorescent study of immunoglobulin-containing plasma cells in tonsil tissue from the same patients (*Østergaard* 1975)

TABLE 3. *Serum Ig levels in the Patients before and after Tonsillectomy*

	IgA IU/ml	IgG IU/ml	IgM IU/ml	IgE IU/ml
Before tonsillectomy				
Range	18–131	70–216	57–356	8–907
Mean	65	135	119	55
After tonsillectomy				
Range	10–124	92–206	61–240	10–600
Mean	59	127	102	54
P =	<0.01	>0.05	<0.01	>0.20

patients with a mean of 65 IU/ml, a difference which, compared with a mean of 103 IU/ml in the controls, was highly significant ($p<0.001$). After the operation, IgA decreased to a mean of 59 IU/ml, and the difference from the pre-operative serum IgA levels was significant ($p<0.01$).

The level of serum IgG in the patients was within the same range as that in the controls and did not decline after tonsillectomy (Tables 2 and 3). Serum IgM was within the same range as that in the controls before tonsillectomy (Table 2) but after the operation this immunoglobulin decreased significantly compared with the pre-operative level ($p<0.01$, Table 3).

As regards serum IgE levels, the observations were very irregularly distributed, but tested by the Mann-Whitney Rank Sum test using 95 per cent confidence limits, the pre-operative mean was significantly reduced as compared with the controls ($p<0.01$). A comparison between the pre- and postoperative means of IgE levels in the patients did not reveal any significant difference ($p>0.20$, Table 3).

Saliva immunoglobulin levels are listed in Table 4. Saliva IgA was demonstrated in all the patients except two before and after tonsillectomy and it was found in all the controls. The mean of the levels observed in the patients before tonsillectomy was 3.7 IU/ml and in the controls 4.9 IU/ml and the difference was significant ($p<0.025$).

Saliva IgG was found in 40 of the patients before and after tonsillectomy and in 39 of the controls. The pre-operative IgG levels did not differ from the levels in the controls ($p>0.1$).

Saliva IgM was only found in measurable amounts in 5 of the controls and it was not demonstrated in the patients.

Comparison of serum- and saliva IgA in patients and controls in the various age groups. It appears from Fig. 1 that the mean of *serum IgA* in patients four to eight years of age was low and the difference from serum IgA levels in age-related controls was significant ($p<0.001$). Conversely serum IgA in the older patients was within the same range as that in the older controls ($p>0.1$).

Saliva IgA levels were also low in the four to eight year old patients, and, compared with saliva IgA in the younger controls, the difference was significant ($p<0.0125$). Such difference was not demonstrated in an at

TABLE 4. *Saliva IgA and IgG Levels in the Controls and in the Patients before Tonsillectomy*

	IgA IU/ml	IgG IU/ml
Patients		
Range	0–13.8	0.09–0.92
Mean	3.7	0.16
Controls		
Range	0.54–40.5	0.09–0.9?
Mean	4.9	0.16
P =	<0.025	>0.1

TABLE 1 *Absence of Ig-containing Plasma Cells in Tonsillar Sections Related to Age and Sex of Patients*

Age (years)	Sex	Total number	Absence of plasma cells IgG	IgA	IgM	IgE	Combined IgA/IgE deficiency
4–9	M	15	2	6	1	9	5
4–9	F	13	1	5	1	10	4
10–15	M	4	0	1	0	3	1
10–15	F	20	0	4	0	9	3
Total		52	3	16	2	31	13

sera before and three to six months after tonsillectomy and on control sera. The results are expressed in U/ml. ASO-titres exceeding 125 U/ml were considered elevated.

Immunofluorescent studies had been performed earlier on the same patients. The rabbit anti human-IgG, IgA, IgM and IgE sera were purchased from Behringwerke AG, Germany. Details concerning the technique, the characteristics of the conjugates, and the fluorescence microscope used have been published earlier (*Ostergaard* 1975).

Statistical evaluation of the results was, in the case of unpaired observations, performed by the Mann-Whitney U test. For paired observations the Wilcoxon test was used. In a few instances a chi-square test was done. The confidence limits of the observations were chosen at the 5 per cent level of significance. P values ≤0.05 were considered significant.

RESULTS

Examination of patients after tonsillectomy. The patients were seen again for follow up three to six months after tonsillectomy. Most of the patients (or their parents) told spontaneously that the tonsillectomy had resulted in a decreased tendency to develop sore throat. Only three patients still had complaints of a recurring sore throat with fever. In one patient, small tonsillar remnants were present, but showed no sign of inflammation. All the patients met for a medical check-up and were found healthy.

Immunofluorescent studies of tonsil tissue. The main results of the immunofluorescent studies are listed in Table 1. It appears from the table that IgE fluorescing plasma cells could not be demonstrated in 31 (60 per cent) of the patients, and IgA fluorescing plasma cells were lacking in 16 (30 per cent). IgA as well as IgE plasma cells were found to be lacking in 13 (25 per cent). Lack of IgE fluorescing plasma cells was mainly observed in the younger patients. Lack of IgG and IgM plasma cells, respectively, was only demonstrated in 3 and 2 of the patients.

Serum immunoglobulin levels in the patients and the controls are listed in Tables 2 and 3. Serum IgA was low in many of the

TABLE 2 *Serum Ig-levels in the Controls and in the Patients before Tonsillectomy*

	IgA IU/ml	IgG IU/ml	IgM IU/ml	IgE IU/ml
Patients				
Range	18–131	70–16	57–356	8–907
Mean	65	135	119	35
Controls				
Range	45–181	51–228	61–192	10–490
Mean	103	132	118	64
P =	<0.001	>0.20	>0.50	<0.01

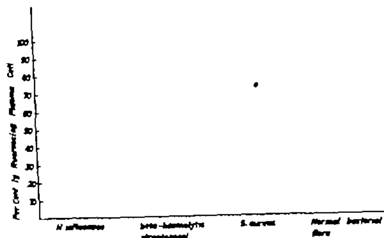


Fig. 2. Correlation between Ig-positive plasma cells in tonsil tissue and bacterial types isolated from the same tissue.
× IgA fluorescing plasma cells   o IgE fluorescing plasma cells.

180 U/ml. However, in 9 such patients, the ASO-titre was less than 125 U/ml, and ASO-antibodies could not be demonstrated at all in 4. Yet, with regard to serum- or saliva IgA levels, any difference between patients with low and high ASO-titres was not observed. A raised ASO was also seen in patients carrying normal bacterial flora (mean titre 175 U/ml), while patients with *H. influenzae* and *S. aureus* had a low titre (60 U/ml and 90 U/ml, respectively).

As a rule, ASO was less than 125 U/ml in most of the controls, but in 12 it was raised considerably. Mean ASO in the patients was 150 U/ml and in the controls 100 U/ml; the difference between patients and controls was significant ($p<0.05$).

Correlation between serum- and saliva IgA levels, Ig-positive plasma cells and bacterial culture types. A low IgA content in serum and saliva, lack of IgA and IgE fluorescing plasma cells in tonsillar tissue, and the carrier rate of pathogenic bacteria seemed to be related in many of the patients and therefore, these observations were compared. The results are listed in Table 5 and Fig. 2. Table 5 shows that mean serum IgA in patients carrying *H. influenzae* was only 59 IU/ml which, compared with a mean serum IgA of 106 IU/ml in age related controls, was significantly reduced ($p<0.01$). By way of comparison, a significant difference between patients with beta-haemolytic streptococci and their age-related controls was also demonstrated ($p<0.01$). Conversely serum IgA did not differ significantly in patients carrying *S. aureus* and normal bacterial flora, as compared with the controls ($p>0.1$ respectively).

With regard to *saliva IgA* and bacterial culture types, no significant difference between patients and controls was observed ($p>0.1$ for beta-haemolytic streptococci and *H. influenzae* respectively).

Fig. 2 shows that a considerable lack of Ig-fluorescing plasma cells in the tonsils was connected with carriage of beta-haemolytic streptococci and *H. influenzae*. A significant difference between lack of IgA plasma cells and carriage of beta haemolytic streptococci and *H. influenzae* on the one hand, and carriage of *S. aureus* and normal bacterial flora, on the other was demonstrated ($\chi^2 = 7.101$, $p<0.01$). There was no such correlation between lack of IgE fluorescing plasma cells and bacterial culture types ($\chi^2 = 3.561$, $p>0.05$).

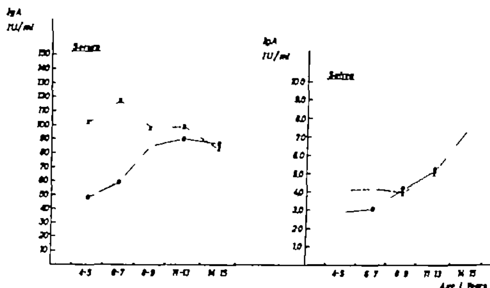


Fig 1 Mean serum and saliva IgA levels in the patients and in the controls with regard to age.
×--×--× Controls o—o—o Patients.

tempt to correlate saliva IgA in the older patients and their age related controls ($p > 0.50$)

Isolation of pathogenic bacteria from tonsil tissue Eleven patients harboured *Haemophilus influenzae* in their tonsils, and 6 of the *H influenzae* strains were of capsular type b. Twenty four patients harboured beta haemolytic streptococci 7 patients *Staphylococcus aureus* and the remaining 10 patients had normal bacterial flora Among the beta haemolytic streptococcal strains 16 belonged to Lancefield's group A, type 4 (1 strain) type 12 (14 strains) and type 22 (1 strain) 3 belonged to Lancefield's group B 2 to group C and 3 to group G Pneumococci were isolated from two patients with *H influenzae* and *S aureus* were found together with *H influenzae* and beta haemolytic streptococci in several patients.

The number of colonies of the mentioned potential pathogens were in all cases several hundreds, up to many thousands, thus not representing occasional contaminants.

ASO-titres showed a wide range in the patients and in the controls. Generally ASO would be raised in patients carrying beta haemolytic streptococci with a mean titre of

TABLE 5 *Correlation between Serum IgA Levels and Bacteria in the Patients and Serum IgA Levels in Age-related Controls*

Bacteria		IgA IU/ml Patients	IgA IU/ml Controls	P =
Beta-haemolytic streptococci	Range Mean	18–156 59	56 181 106	<0 01
Haemophilus influenzae	Range Mean	31–116 51	67 165 112	<0 01
Staphylococcus aureus	Range Mean	43–126 80	67 148 87	>0.1
Normal bacterial flora	Range Mean	45–96 86	41–146 91	>0 1

patients throats did not differ from that in healthy persons. The latter observation is not in agreement with a study by *Kalbak* (1942) who estimated a carrier rate of beta-haemolytic streptococci to 67 per cent in 45 patients with chronic tonsillitis. Therefore, the carrier rate of beta haemolytic streptococci of 46 per cent in the present study is presumably more indicative of patients with chronic tonsillitis.

In view of the postulated function of IgA in secretions (*Gibbons & Van Houte* 1971) the decreased level of saliva IgA in patients comprised in the present series may promote the tendency of pathogenic bacteria to adhere to the tonsil mucosa and colonize in the tonsillar lacunae. Furthermore, the lack of IgA plasma cells in the tonsils in many of the patients supports the view that IgA prevents penetration of microorganisms through the epithelial surface, secondarily establishing an acute inflammation of the tonsils. This hypothesis is favoured by the demonstrated low IgA levels in the younger patients in this study but did not explain the frequent attacks of tonsillitis observed in the older patients, and no difference in attacks rate or duration of symptoms within the age groups was observed this information was given retrospectively by the patients (or their parents) which may imply a certain risk of inaccuracy. Maybe the IgA deficiency represents a transient, rather than a more persistent deficiency in the children and, in an attempt to test this hypothesis, a two year follow-up of the younger patients presented here is being conducted.

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REFERENCES

Alarnas I. M. Parallel study of the character of microflora of tonsillar lacunae, titers of streptococcal antibodies and the results of intradermal tests with bacterial allergens in patients with chronic tonsillitis. Vestn. Oto. Rino. Laring 31 64–69 1969

Ammann A J & Hong, R. Selective IgA deficiency Presentation of 30 cases and a review of the literature. Medicine 50 223–233 1970.

Ammann, A J., Roth J & Hong R.: Recurrent sinopulmonary infections, mental retardation, and combined IgA and IgE deficiency J Pediat. 77 802–804 1970.

Ammann, A J Cain, W A Ishizaka, K., Hong, R. & Good R A Immunoglobulin E deficiency in ataxia-telangiectasia. New Engl. J Med. 281 469–472, 1969

Bardare M Cislaghi, G U & Z ob G Possible relationships between IgA deficiency and recurrent respiratory infections. Helv Paedat. Acta 2 173–178, 1971

Bellanti J A Biologic significance of the secretory gamma A globulins. Pediatrics 48 715–729 1971

Buckley R H., Dee S C & O'Fallon, W M.: Serum Immunoglobulins II. Levels in children subject to recurrent infections. Pediatrics 42 50–60 1968.

Cain, W A., Ammann A J Hong, R Ishizaka, K & Good R. A IgE deficiency associated with chronic sinopulmonary infection. J Clin. Invest. 48 12a–13a, 1969

Cornfield D & Hubbard J P A four-year study of the occurrence of beta-haemolytic streptococci in 64 school children. New Engl. J Med. 264 211–215 1961

Donovan R. & Soothill, J F Immunological studies in children undergoing tonsillectomy Clin. exp. Immunol. 14 347 357 1973

Gibbons R. J & Van Houte J.: Selective bacterial adherence to oral epithelial surfaces and its role as an ecological determinant. Infect Immun. 3 567–572, 1971

Johansson S G O Bennich, H & Wide L.: A new class of immunoglobulin in human serum. Immunology 14 265–272, 1968.

Kalbak K Experimentelle og kliniske undersøgelser over O-streptolysin og forekomsten af O-streptolysin serum. Ed. by Ejnar Munksgaard, Copenhagen, 1942, p. 84

Laurell, C.-B Electroimmuno assay Scand. J Clin Lab. Invest., suppl. 124 29 21–31 1972.

Master P L., Brumfitt W & Mendez R. L.: Bacterial flora of the upper respiratory tract in Paddington families. Brit Med. J 1 1200–1205 1958.

Ogra, P L. Effect of tonsillectomy and adenoidectomy on nasopharyngeal antibody response to polio irus. New Engl. J Med. 284 59–64 1971

South M A Cooper M D Wollheim F A & Good R. A The IgA system II. The clinical significance of IgA deficiency Studies in pa-

DISCUSSION

The most interesting finding in this study was a significantly reduced level of serum- and saliva IgA in the patients as compared with age related controls. Recovery of *Haemophilus influenzae* and beta haemolytic streptococci was found to be connected with low serum IgA and lack of IgA fluorescing plasma cells in tonsil tissue. The low serum- and saliva IgA levels were only demonstrated *in the younger children in this study* whereas the older patients had normal serum and saliva IgA levels.

The significance of IgA in the defence of mucous membranes against infections has been documented repeatedly (*Buckley et al* 1968 *Ammann & Hong* 1970 *Bardare et al* 1971, *Bellanti* 1971) and there are also some indications of a probable connection between isolated IgE deficiency or combined IgA/IgE deficiency and recurrent respiratory infections (*South et al* 1968 *Ammann et al* 1969 *Cain et al* 1969 *Ammann et al* 1970) Therefore, studies of IgE in serum and tonsillar tissue were included in this investigation but although deficiency in serum IgE was demonstrated in the patients, the observations were very irregularly distributed and, in addition, the marked lack of IgE plasma cells in the tonsils did not seem to be connected with the carriage of pathogenic bacteria

The role of IgA in the defence of mucous membranes is not yet fully understood and many apparently healthy persons have no detectable IgA in their serum or secretions (*Johansson et al.* 1968) IgA is predominantly an immunoglobulin operating on mucosal surfaces, but has not generally been found to mediate bactericidal activities. Rather IgA acts on mucosal surfaces by preventing microorganisms from adhering to these surfaces, and the degree to which they adhere influences the extent to which they colonize and penetrate the epithelial membranes (*Gibbons & Van Houte* 1971)

Investigations partially resembling this study have appeared earlier *Ogra* (1971) reported that nasopharyngeal antibodies to oral polio vaccine might be significantly decreased in some children after tonsillectomy and suggested that this was due to the removal of important secretory IgA producing lymphoid tissue. *Ogra's* findings were not supported by *Veltri et al* (1972) who in 17 children undergoing tonsillectomy and adenoidectomy demonstrated a rise in *serum* antibodies to different viral strains 3 to 12 months after tonsillectomy Furthermore, the same authors showed a raised serum IgA and IgG in their patients before tonsillectomy and concluded that this was due to the harbouring of pathogenic bacteria. IgG declined in serum after operation on their patients, but these IgG levels were within the normal limits applying to an age related control group

IgA and IgM decreased significantly after tonsillectomy in the patients in this study The patients were already deficient in IgA before tonsillectomy Therefore, the observed decrease in these immunoglobulins was probably due to the removal of chronically infected organs rather than to removal of important lymphoid tissue. The findings presented are in latter agreement with an investigation by *Donovan & Soothill (1973)* who demonstrated low serum IgA levels in 36 children undergoing tonsillectomy and, in addition, found a relationship between low IgA in serum and successful cultivation of *H influenzae* but not beta haemolytic streptococci, from the patients throats

The significance of the bacteria recovered from the tonsils in more than two thirds of the patients in this study is difficult to evaluate. It is a well-known fact that many healthy persons are carriers of the bacteria demonstrated, but usually not to such a degree as in the patients presented here. The normal carrier rate of beta-haemolytic streptococci and *H influenzae* among healthy school children has been estimated to 3 and 12 per cent respectively in several studies (*Masters et al* 1958 *Cornfield & Hubbard* 1961 *Turk* 1963) In a study of 66 patients with acute and chronic tonsillitis, *Aksnoe* (1969) found that the bacterial flora in the

patients throats did not differ from that in healthy persons. The latter observation is not in agreement with a study by *Kalbak* (1942) who estimated a carrier rate of beta haemolytic streptococci to 67 per cent in 43 patients with chronic tonsillitis. Therefore, the carrier rate of beta-haemolytic streptococci of 46 per cent in the present study is presumably more indicative of patients with chronic tonsillitis.

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REFERENCES

Alsenes I. M. Parallel study of the character of microflora of tonsillar lacunae, titers of streptococcal antibodies and the results of intradermal tests with bacterial allergens in patients with chronic tonsillitis. Vestn. Oto Rino. Laring. *31* 64–69 1969

Ammann A. J. & Hong, R. Selective IgA deficiency. Presentation of 30 cases and a review of the literature. Medicine *50* 223–235 1970.

Ammann, A. J., Roth J. & Hong, R. Recurrent sinopulmonary infections, mental retardation, and combined IgA and IgE deficiency. J. Pediat. *77* 802–804 1970.

Ammann A. J., Cain W. A., Ishizaka K., Hong R. & Good R. A.: Immunoglobulin E deficiency in ataxia-telangiectasia. New Engl. J. Med. *281* 469–472, 1969

Bardar M., Cidoghi, G. U. & Zuccoli G. Possible relationships between IgA deficiency and recurrent respiratory infections. Helv. Paedat. Acta *2* 173–178, 1971

Bellanti J. A. Biologic significance of the secretory gamma A globulins. Pediatrics *48* 715–729 1971

Buckley R. H., Dees S. C. & O'Fallon W. M.: Serum immunoglobulins II. Levels in children subject to recurrent infections. Pediatrics *42* 50–60, 1968

Cain, W. A., Ammann A. J., Hong, R., Ishizaka K. & Good R. A. IgE deficiency associated with chronic sinopulmonary infection. J. Clin. Invest. *48* 12a–13a, 1969

Cornfield D. & Hubbard J. P. A four-year study of the occurrence of beta-haemolytic streptococci in 64 school children. New Engl. J. Med. *264* 211–215 1961

Donovan R. & Soothill J. F. Immunological studies in children undergoing tonsillectomy. Clin. exp. Immunol. *14* 347–357 1973

Gibbons R. J. & Van Houte J. Selective bacterial adherence to oral epithelial surfaces and its role as an ecological determinant. Infect. Immun. *3* 567–572 1971

Johansson S. G. O., Bennich H. & Wide L.: A new class of immunoglobulin in human serum. Immunology *14* 265–272, 1968.

Kalbak K. Experimentelle og kliniske undersøgelser over O-streptolysin og forekomsten af O-streptolysin i serum. Ed. by Ejnar Munksgaard, Copenhagen, 1942, p. 84

Laurell C. B. Electroimmuno assay. Scand. J. Clin. Lab. Invest., suppl. 124 29 21–31 1972.

Master P. L., Brumfitt W. & Mendez R. L. Bacterial flora of the upper respiratory tract in Paddington families. Brit. Med. J. *1* 1200–1205 1958.

Ogra P. L. Effect of tonsillectomy and adenoidectomy on nasopharyngeal antibody response to polio virus. New Engl. J. Med. *284* 59–64 1971

South, M. A., Cooper M. D., Wollheim F. A. & Good R. A. The IgA system II. The clinical significance of IgA deficiency. Studies in pa-

tients with agammaglobulinemia and ataxia telangiectasia. Am. J Med. *44* 168–178, 1968.
Todd E. W Antihaemolysin titres in haemolytic streptococcal infections and their significance in rheumatic fever Brit. J Exp Path. *13* 248–258 1932
Turk D C Naso-pharyngeal carriage of Haemophilus type b J Hyg Camb *61* 247–256 1963
Veltri R W Sprinkle P M Keller S A & Chicklo J M Immunoglobulin changes in a pediatric otolaryngic sample subsequent to T and A J Laryng Otol. *86* 905–916 1972
Weeke B. Carbamylated human immunoglobulin tested by electrophoresis in agarose and antibody-containing agarose. Scand. J Clin Lab Invest. *21* 351–358 1968 a.
Weeke B Quantitative estimation of human immunoglobulins following carbamylation by electrophoresis in antibody-containing agarose Scand J Clin Lab. Invest. *22* 107–113, 1968 b.
Ostergaard P Aa. Presence of immunoglobulin-containing plasma cells in tonsils from tonsillectomized children. Acta path. microbiol. scand. Sect. C, *83* 406–412 1975.

Acta path. microbiol. scand. Sect. C, 84: 299–303, 1976

THE INABILITY OF A DIABETOGENIC VIRUS TO INDUCE DIABETES MELLITUS IN ATHYMIC (NUDE) MICE

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Buschard, K., Rygaard, J. & Lund, E. The inability of a diabetogenic virus to induce diabetes mellitus in athymic (nude) mice. Acta path. microbiol. scand. Sect. C, 84: 299–303, 1976.

The effect of a known diabetogenic M-strain encephalomyocarditis (EMC)-virus in athymic nude mice (lacking the thymus-dependent lymphocyte system) and in heterozygous littermates and homozygous normal mice of the background strain (C57/B16) was investigated. While by 3 weeks 4 out of 4 surviving virus-inoculated littermates and 9 out of 9 inoculated normal mice developed diabetes mellitus, none of the 7 surviving virus-inoculated nude mice became diabetic. Virus was isolated from all inoculated animals, including non-diabetic nude mice. It is concluded that it is the response of the thymus-dependent lymphocyte system evoked by the virus rather than the virus itself that leads to damage to the insulin producing cell.

Key words: Diabetes mellitus; diabetogenic virus; nude mouse; thymus-dependent immune response.

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The present study concerns the effects of a known diabetogenic virus in normal mice and in nude mice. Nude mice are born athymic, thus lacking the thymus-dependent lymphocyte system and, consequently, the ability to raise a cell-mediated immune response (7, 8). Certain viruses, e.g. the encephalomyocarditis (EMC) virus of the picorna group can induce diabetes mellitus in mice (1). During recent years evidence has accumulated that immunologic reactions are involved in the pathogenesis of diabetes mellitus. In laboratory animals a transient diabetic condition can be induced following immunization with homologous pancreatic islets (5). In about 50 per cent of patients with newly developed juvenile diabetes mellitus the lymphocyte migration test, using pooled porcine pancreatic glands as antigen, is positive (6). This suggests the involvement of a T lymphocyte response. The aim of the present study is, by using athymic nude mice, to explore a possible role of the thymus-dependent lymphocyte system in the pathogenesis of the disease.

MATERIALS AND METHODS

Experimental Animals

Three groups of mice were studied:

1) Eighteen 8-week old normal female C57/B16/

TABLE 1 *Numbers of Normal C57/Black 6 Mice Littermates and Nude Mice Surviving and Investigated by Weeks 0-3*

	Normal mice		Littermates		Nude mice	
	virus	no virus	virus	no virus	virus	no virus
Week 0	10	8	5	5	12	10
Week 1	10	8	4	5	10*	10
Week 2	10	8	4	5	7	9
Week 3	9	7	4	4	7	6

* Only 6 out of 10 tested by week 1

BOM. Ten of these were injected with virus, eight with virus-free vehicle.

2) Ten 8-week old heterozygous female litter mates (+/nu, – 5–6 backcross generation of a gene transfer to C57/B16) Five were injected with virus suspension, five with virus-free vehicle.

3) Twentytwo 8-week old homozygous female nude mice (nu/nu – 5–6 backcross generation of a gene transfer to C57/B16) Twelve were injected with virus, ten with vehicle.

All mice were purchased from GL Bomholtgaard Laboratory Animals Breeding and Research Center DK-8680 Ry Denmark. The mice were reared under SPF-conditions, and during experiment kept under conventional conditions at the Royal Veterinary and Agricultural University Copenhagen. The mice were kept two by two in Makrolon® cages, type II. Autoclaved feed pellets (GL Bomholtgaard) and sterile drinking water were supplied *ad lib*

Numbers of mice surviving and investigated by weeks 0 1 2 and 3 are given in Table 1

Virus Virus Assay and Virus Inoculation

The virus employed was an M-strain of EMC-virus with known diabetogenic properties. The strain originated from Craighead's Laboratory (2) and it was received through the courtesy of Dr *Faber Westergaard* The Institute of Medical Microbiology University of Copenhagen The batch of virus employed in this experiment was titrated in Vero cells and has a titer of $10^{5.8}$ $TCID_{50}$/ml. The mice were inoculated intraperitoneally with 0.2 ml of virus suspension containing $10^{\ 0}$ $TCID_{50}$. Control mice were inoculated with suspending medium without virus. After inoculation fecal samples were collected daily from individual mice and the virus contents were controlled by titrations in Vero cells

Metabolic Studies

Glucose tolerance test (GTT) was performed in surviving mice, one week, two weeks, and three weeks following inoculation of virus or control suspension as stated in Table 1 At examination by week 1 only 6 out of 10 nude mice were tested as they tolerate the test procedure less well than do normal mice. Mice were fasted for 24 hours before GTT On testing 2 mg glucose/g BW were administered intraperitoneally Blood samples were taken in 50 µl pipettes from the paraorbital venous plexus, by time zero and 30 and 60 minutes. Blood glucose levels were determined spectrophotometrically following incubation of blood samples with orthotuloidin reagent.

Statistical Studies

The Mann-Whitney U test was used as significance test.

RESULTS

Following virus inoculation diabetes, as demonstrated by an abnormal GTT was induced in all out of 10 normal mice and 4 surviving heterozygous littermates. No sign of diabetes could be demonstrated in any of 7 surviving nude mice The time course of events is described in the following

One Week after Virus Inoculation

Results of glucose tolerance testing are shown in Fig 1 There was no difference between virus inoculated mice and controls and no difference among groups by time zero 30 or 60 minutes ($p > 0.10$) Values of heterozygous littermates are not shown in the figure, but are in full accordance with groups 1 and 3

Two Weeks after Virus Inoculation

Virus inoculated normal mice and heterozygous littermates had significantly higher

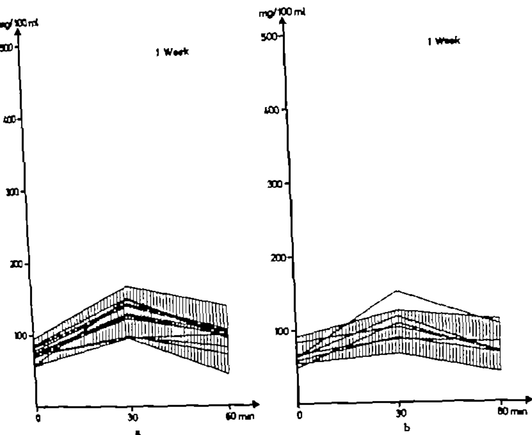


Fig 1 Results of glucose tolerance test after 1 week in normal ( ) and nude (b) mice. Hatched area mean of uninfected control mice ± 2 SD

blood glucose levels after 30 and 60 minutes as compared to controls ($p<0.01$ and $p<0.05$ respectively) In the group of nude mice there was no significant difference between virus inoculated mice and vehicle injected controls ($p>0.10$)

Three Weeks after Virus Inoculation (Fig 2)

In the groups (1 and 2) of immunologically intact normal mice and heterozygous littermates the difference between virus inoculated and vehicle inoculated mice was significant ($p<0.01$) already at time zero. At both 30 and 60 minutes the difference was highly significant, p being <10 In nude mice (group 3) there was no difference between virus injected and vehicle injected mice, neither by time zero, nor by 30 or 60 minutes, $p>0.10$

If virus injected normal and littermate mice were compared with virus injected nudes, there was no significant difference by time zero ($p>0.10$) whereas the difference after 30 and 60 minutes was highly significant ($p<10^{-4}$)

Virus Isolation from the Mice

EMC-virus was demonstrated qualitatively in individual daily fecal samples of all virus-inoculated mice, nude and haired, but from none of the control animals.

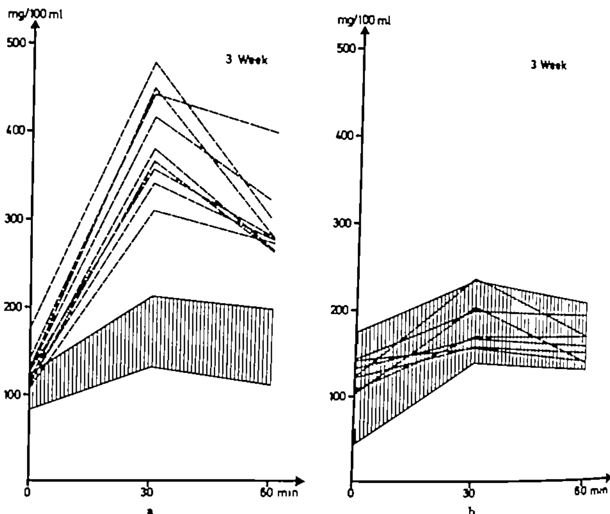


Fig 2 Results of glucose tolerance test after 3 weeks in normal (a) and nude (b) mice. Hatched area mean of uninfected control mice ± 2 SD

DISCUSSION

Our present study has demonstrated that athymic nude mice—as contrasted with normal mice and littermates of the same genetic background—do not develop diabetes following inoculation with the diabetogenic EMC-virus.

A viral etiology of diabetes mellitus has been suggested Experimental studies in laboratory animals have supported this theory It has thus been shown by *Craighead* (2) that EMC-virus can induce diabetes in mice. Other viruses, e.g strains of coxsackie virus, group B type 4 have also been found diabetogenic in laboratory animal models (1) Also studies in man support the idea of a viral etiology of diabetes mellitus. Several case-reports describe the onset of juvenile diabetes in close time relation to virus infection (1) Neutralizing antibodies against coxsackie virus B_4 have been demonstrated in higher titres in patients with recently developed juvenile diabetes than in control populations (3)

As mentioned in the introduction immunologic mechanisms may play an important role in the pathogenesis of this disease. Our investigations with nude mice which lack a thymus-dependent lymphocyte system show that nude mice do not become diabetic in response to EMC-virus. Normal mice of the background strain and heterozygous litter mates all develop manifest diabetic GTT This points to the thymus-dependent lymphocyte system as an essential factor in diabeto-

genesis. Virus infection in itself does not seem to be harmful to the insuline producing cells. The exact mechanism by which the T-dependent lymphocyte system participates remains to be explained. It is possible that T lymphocytes exert killer activity against the virus transformed cell or against shared antigenic determinants of the virus and the cell surface. The demonstration of lymphocytic infiltration of the Islets of Langerhans in patients with juvenile diabetes mellitus (4) supports this point of view. Lymphocytic infiltration of islet tissue is also seen in mice with virus induced diabetes (1). However the T lymphocyte may also function as a helper cell in production of humoral antibodies, reactive with diabetogenic viruses or altered cell surfaces. Current studies in our laboratory are aimed at elucidating these problems.

Our present observation strengthens the link between diabetogenesis and thymus-dependent lymphocyte function.

We wish to thank the *Clinical Computer Laboratory at Rigshospitalet*, Copenhagen, for statistical support. Dr. *Wilhem P. Weidens* kindly read our manuscript.

REFERENCES

1. *Craighead J. E.:* The role of virus in the pathogenesis of pancreatic disease and diabetes mellitus. Progr. med. Virol. *19*: 161–214 1975
2. *Craighead J. E. & McLane M. F. R.:* Diabetes mellitus. Induction in mice by encephalomyocarditis virus. Science *162*: 913–914 1968.
3. *Gamble D. R. Kinsley M. L., Fitzgerald M. G., Bolton R. & Taylor K. W.:* Viral antibodies in diabetes mellitus. Brit. Med. J. 3: 627–630 1969
4. *Gepts W.:* Pathologic anatomy of the pancreas in juvenile diabetes mellitus. Diabetes *14*: 619–633 1965
5. *Nerup J. Andersen O. Orsted Bendixen G. Egeberg J. Gunnarsson R. Kromann H. & Poulsen J. E.:* Glucose intolerance and islet damage in mice immunized with homologous endocrine pancreas. Horm. Metab. Res. *6*: 173–175 1974
6. *Nerup J. Andersen O. Orsted Bendixen G., Egeberg, J. & Poulsen J. E.:* Antipancreatic cellular hypersensitivity in diabetes mellitus. Diabetes 20: 424–427 1971
7. *Pantelouris E. M.:* Absence of thymus in a mouse mutant. Nature *217*: 370–371 1968.
8. *Rygaard J.:* Thymus & Self Immunobiology of the mouse mutant nude. F.A.D.L., Copenhagen, and J. Wiley & Sons, London, 1973

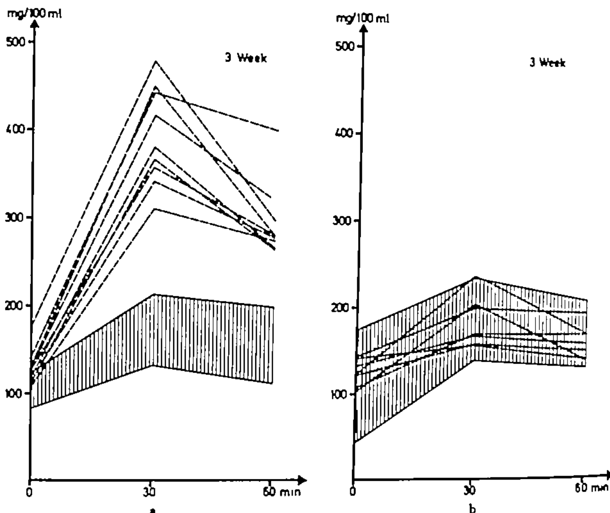


Fig 2 Results of glucose tolerance test after 3 weeks in normal (a) and nude (b) mice Hatched area mean of uninfected control mice ± 2 SD

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ABO antigens in the graft, but most often it has been ascribed to the presence of relevant cytotoxic HLA-A and B antibodies in the recipient's serum. A few cases of hyperacute rejection in the absence of preformed antibodies have been communicated (23–26) but such cases seem to be rare. On the other hand, one case has been reported which exhibited weak anti-HLA A2 antibodies and the patient was successfully transplanted with a HLA-A2 positive kidney (10).

This report describes a case of hyperacute rejection of a HLA A and B full house identical, unrelated necrokidney which cannot immunologically be explained by known, relevant preformed antibodies in the recipient, but possibly by preformed and rapidly mobilized effector lymphocytes as revealed by the direct Cell Mediated Lympholysis (CML) test.

METHODS

a) HLA Typing and Crossmatching

were performed according to the *Kissmeyer Nielsen & Kjerbye* lymphocytotoxic technique (12) including incubation for 30 minutes as well as for 60 minutes.

b) Direct Cell Mediated Lympholysis (Direct CML) Test

was performed as described in detail elsewhere (7, 8, 14). In brief, lymphocytes from the recipient to be investigated for cytotoxic capacity (i.e. effector lymphocytes) were confronted with Cr 51 labelled, freshly isolated lymphocytes (i.e. targets) either from the donor or from healthy randomly selected individuals.

This test was performed in heat inactivated (56 C, 30 min) indifferent serum.

Chromium release due to lympholysis is expressed as Cr 51 release percentage and calculated by the formula

$$\frac{\text{(experimental – spontaneous) cpm}}{\text{(maximum – spontaneous) cpm}} \times 100 \text{ per cent}$$

Maximum release was defined by repeated (×3) freeze thawing of target cells alone, while spontaneous release was defined by the release from target cell cultures incubated alone. All combinations were performed in duplicates.

c) Lymphocyte Antibody Lymphocytolytic Interaction (LALI) Test

is an investigation into the presence of complement independent antibodies in the recipient which in concert with unprimed lymphocytes are active against donor lymphocytes. In principle, LALI is a direct CML test performed in heat inactivated (56 C, 30 min) recipient serum (final dilution 1:4). Lymphocytes from three healthy randomly selected individuals were included as LALI-effectors (i.e. unprimed lymphocytes).

d) Determination of Complement (C) Components

C1q-component was identified by radial immunodiffusion according to *Mancini et al.* (19).

Quantitative references were sera from 10 normal, healthy individuals.

C3-proactivator (C3PA) was identified by radial immunodiffusion according to *Mancini et al.* (19).

For quantitation, standard references provided by Behringwerk (Germany) were included.

C3-component was identified and quantitated by rocket immunoelectrophoresis according to *Laurell* (17).

All antibodies were purchased from Behring werke (Germany).

e) B-lymphocytes

were enumerated by the direct immunofluorescence technique as described by *P. pernochall et al.* (24) with minor modifications.

A total of more than 200 cells were counted.

f) T-lymphocytes

E-rosettes were prepared as described by *Bach* (1) with minor modifications.

A total of more than 200 cells were counted in Bürker Turk haemocytometer.

MATERIAL

The material comprises one patient transplanted for the third time with a HLA-A and B compatible renal allograft.

Case History Prior to the Actual Transplantation

The patient is an 18-year-old male individual who has been suffering from glomerulonephritis since the age of two (1955). A percutaneous renal biopsy made when he was 11 years old showed focal proliferative glomerulonephritis. He has been treated regularly with haemodialysis since the age of 15 (1973). Due to hypertension, bilateral nephrectomy was performed at the age of 17 (1974). The kidneys were small and histological examination revealed chronic glomerulonephritis. Prior to the actual transplantation, two allotransplantations using cadaver-kidneys, have been unsuccessfully performed. The patient received a total of 34 units of blood before the actual (3rd) transplantation.

Recipient tissue type: HLA-A2,3,Bw15,w35. Blood group: A Rhesus negative (phenotype: C–,c+,D–,E–,e+).

Acta path. microbiol. scand. Sect. C, 84: 304–312, 1976

HYPERACUTE REJECTION OF A KIDNEY ALLOGRAFT MAY BE CAUSED BY CYTOTOXIC LYMPHOCYTES

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Kristensen, T., Grunnet, N., Madsen, M., Sølling, J., Fjeldborg, O., Olsen, T. Steen & Kissmeyer Nielsen, F. Hyperacute rejection of a kidney allograft may be caused by cytotoxic lymphocytes. Acta path. microbiol. scand. Sect. C, 84: 304–312, 1976.

A case of hyperacute rejection of a kidney allograft is described in relation to the clinical, patho-anatomical and immunological findings. An 18-year-old male was allotransplanted for the third time with a necrokidney from an unrelated HLA A and B, full house identical and bloodgroup ABO identical donor. Serological crossmatches performed with recipient sera harvested before and after transplantation were negative. In spite of this, the kidney suffered hyperacute rejection according to clinical and patho-anatomical criteria. The cellular complement independent cytolytic capacity of recipient lymphocytes drawn before and after transplantation against donor lymphocytes was tested by the direct *Cell Mediated Lympholysis* (CML) test. This test was positive 24 hours after transplantation, whereas it had been negative before this. Lymphocytolysis was enhanced by inactivated recipient serum harvested before *and after transplantation. These findings suggest that hyperacute rejection of a kidney allograft* may be ascribed to presensitized, not necessarily circulating, effector lymphocytes either alone, or in concert with antibody(ies) not disclosed by conventional crossmatching.

Key words: *Hyperacute rejection; cell mediated rejection.*

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Hyperacute rejection of a kidney allograft was originally described by *Kissmeyer Nielsen et al.* (11) and their findings have been confirmed by several other authors (2, 22, 25, 26, 27).

The hyperacute rejection is classically defined (i) *clinically* by the absence or rapid cessation of urine flow at revascularization, a mottled *cyanotic colour* and a floppy consistency of the graft, (ii) *patho-anatomically* by extensive microthrombosis of the glomerular capillaries with subsequent renal cortical necrosis, and (iii) *immunologically* by preformed circulating antibodies active against donor antigens. The kidney is rejected within one or a few hours after the circulation in the recipient has been reestablished.

The phenomenon may be due to ABO antibodies in the recipient and active against

ceased. There was, however still good pulsation in the graft artery and apparently good bloodflow through the graft.

The graft was left for 24 hours and then removed. The appearance of the graft at graftectomy was similar to the appearance 24 hours before.

The immunosuppressive therapy was initiated during the operation, using azathioprine 5 mg/kg. No steroid or antilymphocyte serum was given, and heparin was not administered during or after the operation. The patient had been haemodialysed a few hours before operation.

The patient received one unit of leucocyte poor blood during the operation.

(b) *Patho-anatomical findings.* The graft measured 11 × 7 × 8 cm. The surface was smooth, of a greyish-red colour with a few petecchial haemorrhages. On the cut surface the cortex measured 7 mm, it was of a light grey colour contrasting against the dark red medulla. All larger arteries were patent. By light macroscopy a nearly total cortical necrosis was seen, demarcated against narrow vital subcapsular and juxtamedullary zones by a leucocyte infiltrate of medium degree. Despite the necrosis, it was easily seen that most glomerular cross sections contained capillary thrombi (Fig 1) These were clearly visualized by staining with phosphotungstic acid haematoxylin. The small arteries were most often patent, in some places however a thin layer of fibrin was covering the intima. There was no mononuclear infiltrates in the interstitial tissue By immunofluorescence technique, neither immunoglobulins nor complement were revealed in the glomeruli or vessels. Using anti-fibrinogen, a strong fluorescence was seen in many glomerular capillaries and arterioles as well as in some small arteries Thus the pathoanatomical picture was that of a disseminated arteriolar and glomerular microthrombosis with early cortical necrosis, a pattern fully in concordance with the diagnosis of a hyperacute rejection.

(c) *Immunological findings.* Tissue typing of the recipient as well as the donor was performed by the same laboratory using an identical set of antisera. No discordant results were obtained. Thus, both recipient and donor are blank in the HLA-C series.

After the first renal allotransplantation (1973) multispecific complement dependent lymphocytotoxic antibodies as well as a Rhesus anti-D antibody demonstrable only with papain technique were identified. The multispecific cytotoxic antibodies have continously been present (titres 2-128) while the Rhesus anti-D antibody (titre initially 32) disappeared and could not be identified over a period of 18 months (1974–1975)

After the third (and actual) renal allotransplantation (1975) the Rhesus anti-D antibody reappeared as well as a very strong anti-E antibody (titres saline 37° 128, indirect Coombs 128, papain 2048) Crossmatchings were performed using three different, recipient sera 1) an older serum known to contain lymphocytotoxic antibodies as judged by screening against a panel of 25 HLA different unrelated individuals. This serum reacted positively with 24 out of 25 cells. 2) serum drawn from the recipient immediately before transplantation. 3) serum drawn from the patient immediately before graftectomy (i.e. 24 hours after the transplantation) All these sera have been tested twice against donor lymphocytes and the crossmatch was always fully negative

Thus, there is no indication of the presence in the recipient of complement dependent, lymphocytotoxic antibodies directed against donor lymphocyte antigens.

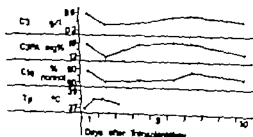


Fig 2 Course of recipient complement concentrations and body temperature in relation to the communicated case of hyperacute rejection of a kidney allograft

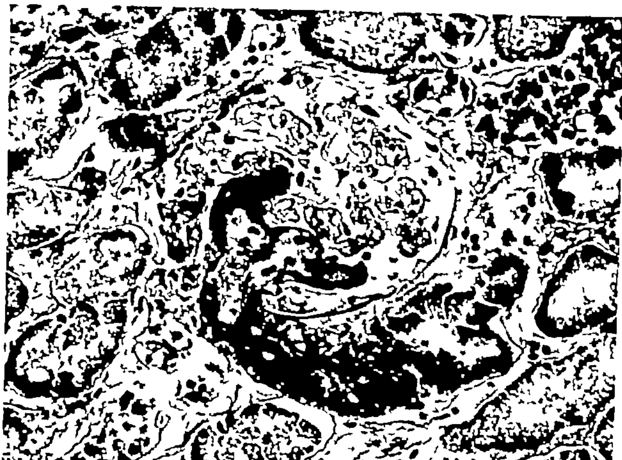


Fig 1 Glomerulus from the hyperacute rejected graft. There is microthrombosis in an arteriole and some glomerular capillary loops (PTAM-stain) × 370

Renal allotransplantation I was performed at the age of 15 (1973) The donor was HLA A2 3 Bw15 w35 (blood group A Rhesus type unknown) and thus the HLA match was an A match (full house HLA A and B identity) The serological crossmatch was negative.

The graft never functioned and was removed 36 days after transplantation.

Renal allotransplantation II was performed at the age of 16 (1974) The donor was HLA A2 3 Bw35 Cw4 (blood group A, Rhesus negative (D−)) HLA matching for HLA A and B series showed compatibility (the recipient possesses one antigen which cannot be identified in the donor) Serological crossmatch was negative

The graft did not function at any time after transplantation and was removed after 51 days.

RESULTS

The third renal allotransplantation was performed at the age of 18 (1975) The donor was HLA A2,3 Bw15 w35 (blood group A Rhesus positive (D+)) thus an A match.

Donor died from headinjury and was pretreated with manitol, heparin, and chlorpromazine Warm and cold ischaemia time were 15 minutes and 16 hours, respectively The kidney was perfused without problems, with 300 ml of cold Collins solution and kept at 4–6 C by ice Perfusion machinery was not used for preservation. Both kidneys revealed a few small subcapsular bleedings. Freezemicroscopy of the contralateral kidney (which was not used) showed normal parenchyma except for a few small bleedings.

(a) *Clinical findings* Transplantation was technically uncomplicated On removal of the arterial forceps, the graft assumed normal colour and tonus, ureter peristalsis was observed and a few drops of urine were produced 15 minutes later the graft assumed a blueish colour starting disseminated but soon becoming generalized. The renal parenchyma was floppy and the production of urine

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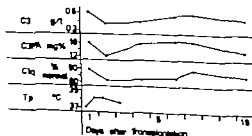


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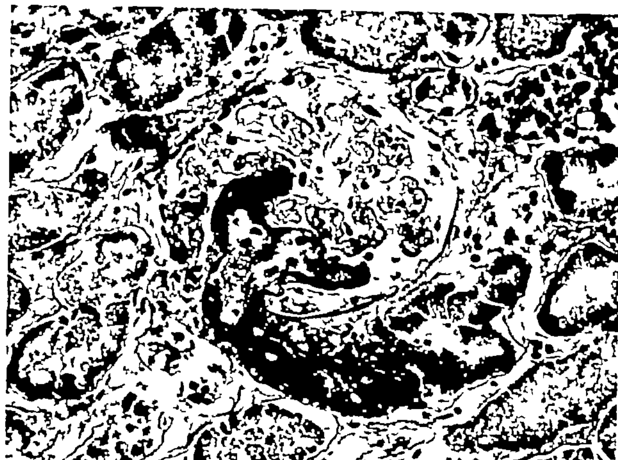


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TABLE 2 A. *Direct Cell Mediated Lympholysis (CML) in relation to a Case of Hyperacute Rejection of a Kidney Allograft*

Effector lymphocytes	Target lymphocytes: Donor lymphocytes (%)	Lymphocytes from graft perfusate (24 h after RAT) (%)	Normal control target (%)
Recipient lymphocytes harvested before RAT	1.6	1.9	3.1
Recipient lymphocytes harvested 24 h after RAT	*22.4*	−3.9	*10.8*
Lymphocytes from graft perfusate (24 h after RAT)	*16.8*	−1.6	8.5
Normal control A	0.7	0.4	−0.3
Normal control B	−2.9	−2.4	−0.6
Normal control C	−1.7	−0.3	−0.5

TABLE 2 B. *Lymphocyte Antibody Lymphocytolytic Interaction (LALI) in Relation to a Case of Hyperacute Rejection of a Kidney Allograft*

Effector lymphocytes	Target lymphocytes: Donor lymphocytes (%)	Lymphocytes from graft perfusate (%)	Lymphocytes isolated from minced graft (%)	Normal control target A (%)	
Lymphocytes from graft perfusate	*26.8*	−8.8	0.8	*28.9*	Recipient serum
Normal control A	3.2	−9.1	0.9	6.7	harvested before RAT
Normal control B	5.9	2.2	−3.6	6.4	Dilution 1:4
Normal control C	2.9	0.3	−1.4	3.2	
Lymphocytes from graft perfusate	*29.4*	−6.4	*16.7*	*27.6*	Recipient serum
Normal control A	3.5	−7.2	−11.0	6.0	harvested after RAT
Normal control B	*10.5*	3.0	0.7	3.5	Dilution 1:4
Normal control C	7.4	−2.4	−3.3	4.7	

The CML- and LALI-capacity of recipient lymphocytes and serum in relation to hyperacute rejection of a kidney allograft. It is seen that recipient lymphocytes harvested prior to transplantation (A) do not exhibit cytolytic capacity against donor target lymphocytes. By contrast, recipient lymphocytes harvested after transplantation (24 h) exhibit significant cytolytic capacity against donor target cells as well as against target cells from a normal, unrelated control subject. The same pattern is seen if lymphocytes harvested from graft perfusate (and presumably being recipient lymphocytes) are used as effectors.

LALI testing (B) using complement inactivated recipient serum (final dilution 1:4) harvested prior to or after transplantation provides a comparable picture but exhibits higher release percentages and includes positive scoring on lymphocytes isolated from a graft homogenate.

For further details cf. text.

Italics = positive results.

TABLE 1 *Results of T and B-lymphocyte Enumeration. All the Listed Figures Were Determined on One Occasion*

Source	% B-lymphocytes (SD = 3 0)	% T-lymphocytes (SD = 5.8)	% "O"-cells* (SD = 6.5)
Recipient PBL§ *before* RAT†	16 0	63 6	20.5
Recipient PBL *after* RAT	62.8	27.2	9.9
Lymphocytes from graft perfusate (24 h after RAT)	66 7	32.9	0.5
Normal control A (PBL)	12 6	67 1	20.2
Normal control B (PBL)	15 6	59 9	24.5
Normal control C (PBL)	15.2	52.9	31 9

* "O"-cells 100% (% B-lymphocytes + % T lymphocytes)
§ PBL peripheral blood lymphocytes.
† RAT renal allotransplantation.

Some complement activation and consumption however took place during the rejection period The complement components C3 C3PA and C1q were determined at regular intervals before during and after rejection Results are given in Fig 2

The serum-complement of the patient is usually found to be within the normal range (i.e. C3 0 65–1 10 g/l C3PA ~ 20 mg per cent, C1q 90–110 per cent or normal sera) During the first 24 hours after RAT a marked fall in the concentrations of these complement components was observed These concentrations reverted to normal within a few days after graftectomy

In order to investigate whether complement independent preformed and circulating effector lymphocytes of T-cell origin or complement independent LALI antibodies directed against donor antigens were present in the recipient before and after the transplantation defibrinated blood was prepared from the recipient immediately before transplantation and immediately before graftectomy

Upon removal, the graft was also perfused with approximately 50 ml of isotonic saline and lymphocytes were harvested. (It should be noticed that the yield of lymphocytes from these 50 ml saline was extremely high, approximately 5 × 10⁷ cells which equals the number of cells usually harvested from 50 ml of whole blood)

Lymphocytes isolated from these samples were tested to estimate the T and B-cell amount and hence tested in the direct CML and LALI tests. Results are given in Table 1 and 2.

Firstly it is seen (Table 1) that the relative amounts of T and B-lymphocytes in the blood samples harvested after transplantation as well as in the graft perfusate were reversed as compared with the blood sample taken before transplantation and compared with blood samples from three random control individuals. Secondly (Table 2 A) cells isolated *before* transplantation did not show cytotoxic activity against donor lymphocytes or against the lymphocytes harvested from the graft perfusate or against lymphocytes from one random healthy individual. Thirdly it is seen that lymphocytes drawn from the recipient 24 hours *after* transplantation exhibited a cytotoxic capacity against *donor* lymphocytes as well as against the lymphocytes in the control subject Finally (Table 2 B) it is seen that a similar pattern may be obtained by the LALI test. There is no clear indication of LALI-antibodies in the recipient sera since cytolysis with unprimed LALI-effector lymphocytes from the three random control, (A, B and C) is only observed if B is used (10.5 per cent)

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Normal control A	3.2	-9 1	0.9	6.7	harvested before RAT
Normal control B	3.9	2.2	3 6	6.4	Dilution 1-4
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LALI testing (B) using complement inactivated recipient serum (final dilution 1-4) harvested prior to or after transplantation provides a comparable picture but exhibits higher release percentages and includes positive scoring on lymphocytes isolated from graft homogenate

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Italics = positive results.

DISCUSSION

Our findings indicate that a positive lymphocytotoxic complement dependent, humoral crossmatch is not a conditio sine qua non for the occurrence of hyperacute rejection of a kidney allograft. Furthermore, our findings *seem to indicate that hyperacute rejection* may be ascribed to pre-existing (not necessarily circulating) effector lymphocytes of T-cell origin either alone or in concert with humoral factors.

Thus it seems certain that one or several cellular mechanisms participate in this case of hyperacute rejection It cannot be excluded, however, that complement-dependent antibodies not disclosed by crossmatching also are active Indeed, our data on C3 C3PA and C1q concentrations are suggestive in this respect *Lightbody* & *Rosenberg* have provided suggestive evidence (18) with regard to the significance of complement independent, LALI, antibodies in prospective kidney recipients.

Such antibody(ies) may be active against donor antigens coded for by genes in- or outside the human *M*ajor *H*istocompatibility *C*omplex (MHC) Donor and recipient were known to match fully with regard to HLA A and B series antigens, whereas there are potential mismatches in the *HLA-C* and *D* series. Whether pre-existing antibodies active only against antigens from one or both of these loci may cause hyperacute rejection by themselves is not known

It must be noticed that the recipient was previously immunized against the Rhesus blood group antigen D and that the immunization most probably was caused by transplantation number one. Furthermore that another Rhesus antibody (anti E) was raised by the recipient after the actual (3rd) transplantation It has not been established however whether Rhesus blood group antigens are present in kidney or endothelial tissue.

Consequently our findings obtained by the direct CML test system remain the only direct and positive immunological observation. The negative findings obtained by recipient lymphocytes rescued immediately before transplantation and the clearly positive findings obtained by recipient lymphocytes drawn 24 hours later (either from peripheral blood or graft perfusate) point to the conclusion that pre-existing effector lymphocytes of T-cell origin need not necessarily be circulating in order to cause hyperacute rejection. On the other hand such effector cells must be pre-existing since they cannot be educated within minutes or hours.

The antigens attacked by preformed effector lymphocytes *in vivo* are unknown. In this particular case, it cannot be HLA A and B antigens, since donor and recipient were HLA A and B identical. From *in vitro* studies using CML after cellular sensitization via *M*ixed *L*ymphocyte *C*ultures (MLC) it has been established that the antigens of the HLA A B and C series may act as target determinants both in a qualitative and a quantitative way (3 5 6 9 15 16, 28) while the HLA D antigens are not target determinants (21) Furthermore *in vitro* studies have substantiated the existence of a separate CML determinant locus inside the MHC in close linkage disequilibrium with the HLA B locus (13)

Studies using CML after *in vivo* immunization have provided less conclusive results. *Mawas et al* suggested the existence of a separate CML locus not linked to the HLA (20) *Goldmann et al* were able to demonstrate specific CML activity after bonemarrow transplantation between two unrelated HLA A, B and D ABO and Rhesus identical individuals (4) while our group—in studies of CML activity in relation to renal allotransplantation—were able to find some correlation between positive direct CML and occurrence of acute rejection episodes, but unable to find any consistency between the pattern of HLA antigens mismatched in the transplantation and the direct CML pattern (7 8)

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REFERENCES

1 *Bach, J-F* E aluation of T-cells and thymic serum factors in man using the rosette technique. Transplant. Rev *16* 196–217 1973

2 *Bergentz S-E., Olander R Kissmeyer-Nielsen, F Olsen, T S & Hood B.* Hyperacute rejection of a kidney allograft. Scand. J Urol. Nephrol *4* 143–148, 1970.

3 *Eijsvoogel V P., Bois, M J G J d Meduf C. J M Groot Keey M L. de Koning, C., Rood J J van Leeuwen A. an Tell E. D du & S hellekens P T A.* Position of locus determining mixed lymphocyte reaction (MLR) distinct from the know HL-A loci and its relation to call mediated lympholysis (CML) Histocomp Testing 1972, p. 501–508, 1973

4 *Goldmann, S F., Kristensen T., Grunnet N., Lohrmann H-P & Kissmeyer-Nielsen, F* Education of lymphocytes in vivo following a transient take of matched unrelated bone marrow graft in man. Clin. exp. Immunol 1976 (in press)

5. *Grunnet N Kristensen T Jorgensen F & Kissmeyer-Nielsen F.* Cell mediated lympholysis in man. The impact of SD determinants on in vitro induced lympholysis. Tissue Antigens 4 218–228, 1974

6 *Grunnet N Kristensen, T & Kissmeyer Nielsen, F* Cell mediated lympholysis in man Patterns of killing power in relation to the HL-A system Tissue Antigens 6 205–220 1975

7 *Grunnet N Kristensen T Kornerup H J & Kissmeyer-Nielsen, F* Direct cell mediated lympholysis in man A test of allograft-rejection in human kidney recipients. Tissue Antigens 5 280–285, 1975

8 *Grunnet N & Kristensen T* Direct cell mediated lympholysis by peripheral blood lymphocytes from renal allograft recipients. Scand. J Urol. Nephrol 29 41–44 1975

9 *Grunnet N Kristensen T & Kissmeyer Nielsen F* Cell mediated lympholysis in man. The impact of HLA-C antigens. Tissue Antigens, 1976 (in press)

10 *Hoels W F Morris, P J Bennett R. C., Mortensen, P J & Ting, A.* Leukocyte antigens in renal transplantation VIII. Successful renal transplantation in the presence of positive cross match Med. J Aust. 2 382–384 1969

11 *Kissmeyer-Nielsen F Olsen, S., Petersen, V P sberg & Fjeldborg, O* Hyperacute rejection of kidney allografts, associated with pre existing humoral antibodies against donor cells Lancet ii 662–665 1966.

12. *Kissmeyer-Nielsen, F & Kjerbye K. E.* Lymphocytotoxic microtechnique purification of lymphocytes by floatation. Histocomp. Testing 1967 p. 381–383 1967

13 *Kristensen T & Grunnet N.* Cell mediated lympholysis in man. E idence of separate CML locus within the major histocompatibility complex and an approach to CML typing Histocomp. Testing 1975 p. 835–844 1975.

14. *Kristensen T & Grunnet N.:* Cell mediated lympholysis in man. Technical aspects. Histocomp. Testing 1975 p. 899–907 1975

15. *Kristensen T., Grunnet N & Kissmeyer Nielsen F.* Cell mediated lympholysis in man. Varying strength of HL-A (LA and FOUR) antigens as sensitizing or target determinants. Tissue Antigens *6* 221–228 1975

16. *Kristensen T., Grunnet N & Kissmeyer-Nielsen F :* Cell mediated lympholysis in man. The HL-A third locus antigen AJ (W20) as sensitizing and/or target determinant. Tissue Antigens 6 229 236, 1975

17 *Laurell, C-B* Quantitati estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem. *15* 45 1966.

18. *Lightbody J J & R senberg, J C.* Antibody dependent cell-mediated cytotoxicity in prospecti kidney transplant recipients. J Immunol. *112* 890–896 1974

19 *Mancini, G., Carbonar A O & Heremans J F* Immunological quantitation of antigens by single radial diffusion. Immunochem. *2* 235 1965

20. *Mawas, C., Christen Y Legrand L., Sasportes M & Dausset J.* Cellular and humoral response against determinants other than the classical HL-A specificities. Transplantation *18* 256–266, 1974

21 *Mawas, C., Charmot D & Sasportes M.* Is the LD region of the human MHC a CML target? Histocomp. Testing 1975 p. 855–858, 1975.

22 *Morris P J Williams G M., Hume D M., Mickey M R. & Terasaki P I.:* Serotyping for homotransplantation. XII Occurrence of cytotoxic antibodies following kidney transplantation in man Transplantation *6* 392–399 1968

23. *Morris P J Kincaid-Smith, P., M Kenzie I F C., Mathew, V C & Ting, A.* Leukocyte antigens in renal transplantation. 7 Immediate renal allograft rejection with a negati cross match. Med. J Aust. 2 379–382, 1969

24 *P pamichail, M Brown, J C. & Holborow E. J* Immunoglobulins on the surface of human lymphocytes. Lancet ii 850–852, 1971

25 *Patel R. & Terasaki P I.* Significance of the positi cross match test in kidney transplantation. New Engl. J Med. *280* 735 1969

21

26 *Starzl T E Lerner R Dixon F J Groth C G Brettschneider L & Terasaki P I* Schwartzman reaction after human renal homotransplantation. New Engl J Med 278 642–648 1968
27 *Terasaki P I Trasher D L & Hauber T H* Serotyping for homotransplantation. XIII Immediate kidney transplant rejection and associated preformed antibodies. Advance in Transplantation, p 225–229 1967
28 *Trinchieri G Bernoco D Curtoni S E, Miggiano V C & Ceppellini R.* Cell mediated lympholysis in man. Relevance of HL-A antigens and antibodies. Histocomp. Testing 1972 p 509–519 1973

Acta path. microbiol. scand. Sect. C, 84: 313–318, 1976

THE RELATIONSHIP BETWEEN PREGNANCY, HCS AND B LYMPHOCYTES

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Christiansen, J. Sandahl, Andersen, A. Renard, Osther, K., Pettersen, B., Bach-Mortensen, N. & Lebech, P. E. The relationship between pregnancy, HCS and B lymphocytes. Acta path. microbiol. scand. Sect. C, 84: 313–318, 1976.

The quantitative distribution of the lymphocyte subpopulations (B, T and null) and the serum concentrations of Human Chorionic Somatomammotropin (HCS), immunoglobulins IgG, IgM and IgA, complement component C4 and C1 inactivator were estimated in venous blood samples from 52 women at various stages of pregnancy and compared with a control series of 7 non-pregnant normal women. A significant decline in the B cell percentage during pregnancy was seen. The fall in the percentage of the B lymphocytes was found to be concomitant with the rise in HCS concentration. No significant changes in the other parameters studied were present.

Key words: Human chorionic somatomammotropin; B lymphocytes; pregnancy.

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Recently attention has been drawn towards the immunological mechanisms involved in the protection and survival of the foetus during intra-uterine life (3).

Several authors have demonstrated a reduced mitotic response of pregnant women's lymphocytes to phytohaemagglutinin (PHA) (11, 23, 30). The normal PHA response is generally found to be associated with T lymphocytes. The presence of serum inhibitors in pregnancy is by some authors held to be responsible for the altered PHA response (25, 28) and the interference with Mixed Lymphocyte Culture (MLC) reactions (15, 18, 26).

Recently we have observed a percentual diminution of the B lymphocytes in venous blood drawn from women immediately after the termination of a normal pregnancy (9).

The aim of the present work has been to study the quantitative distribution of lymphocyte subpopulations during pregnancy. Furthermore the serum concentrations of immunoglobulins, C4 and C1 inactivator (C1IA) have been estimated. The results have been related to the serum concentration of Human Chorionic Somatomammotropin (HCS).

MATERIALS AND METHODS

Source of lymphocytes. Venous blood was obtained from 52 women attending the Obstetrical department F, Frederiksberg Hospital for routine control

of their pregnancy All the pregnancies were clinically normal Seven nulliparous, non-pregnant women who did not use hormonal contraception served as controls.

The blood was collected in tubes containing 1 ml ACD (NIH solution A) Separation of the lymphocytes was started within 4 hours. The patients were selected consecutively and the estimation of the B, T and null cells was performed without any previous knowledge of the week of gestation.

Testsera. Rabbit anti human IgG and IgM antibodies were obtained from Dakopatts A/S Denmark The chromatographically purified immunoglobulins were conjugated with fluorescein isothiocyanate (FITC) (1?) Excess of FITC was removed by gel filtration on Sephadex G ?5

Sheep erythrocyte preparation A 1 per cent suspension of washed sheep erythrocytes, stabilized in 5 per cent inactivated human AB serum, was used for identification of T lymphocytes, as described by *Brain & Gordon* (5)

Preparation of lymphocytes for B T and null cell estimation The lymphocytes were isolated by the Isopaque-Ficoll gradient centrifugation method, described by *Bøyum* (7) The lymphocytes were washed once in 5 ml 0.9 per cent saline 250 µl lymphocyte suspension containing approximately 8 × 10⁶ cells/ml were incubated with 500 µl of FITC labelled anti human IgG and anti human IgM antisera for 30 minutes at 37 C. Excess of antisera was removed by 3 washings in 0 9 per cent saline. 250 µl sheep erythrocyte suspension was added to the lymphocytes and incubated at 37 C for 15 minutes. After centrifugation at 800 rev/min for 10 minutes the cells were kept at 4 C for a period of 2 hours and then gently resuspended One drop of the suspension was placed on a slide and sealed

Measurements The lymphocytes were considered spontaneous, rosette-forming cells if 2 or more erythrocytes were attached to the cell At least 100 cells were examined for the rosette-formation. On the same preparation, cytophotometric measurements of 100 nonrosette-forming cells were performed.

The cytofluorimeter consisted of a Leitz MPV I system assembled on a Leitz Orthoplan microscope. The excitation light source was a HBO 200 The lamp housing was bayonette mounted to the PI OEM epi-illuminator port The excitation light passes a 1½ mm BG 12 filter in the lamp housing and a narrow band filter KP 490 in the epi-illuminator port. A K 530 barrier filter excluded nonspecific signals. The determination of a borderline between specific and nonspecific fluorescence has been described by *Osther & Dybkjaer* (22)

As running test a B T and null cell estimation was carried out on donor blood every day in the laboratory Values are expressed as mean of two different measurements on two different slides. Differences in duplicates generally did not exceed five per cent

In agreement with several authors (13 14 24, 27 29) rosette-forming cells were regarded as T lymphocytes and immunoglobulin coated cells as B lymphocytes. Cells with no detectable surface markers were regarded as null cells.

Sero-immunological estimations. The immunoglobulins IgG IgM and IgA the complement component C4 and the natural inhibitor of C1 esterase called C1 inactivator were estimated by rocket immuno-electrophoresis, as described by *Laurell* (19) As antibody containing gel agarose Indubiose A 37 (L Industrie Biologique Française S.A.) was used. Electrophoresis was performed at ?.5 V/cm for 18 hours. Rabbit anti human IgG (antibody content 0.4 mg/ml) IgM (antibody content 0 4 mg/ml) and IgA (antibody content 0 4 mg/ml) were obtained from Dakopatts A/S, Denmark. Rabbit anti human C4 (antibody content 0 1 mg/ml and C1 inactivator (antibody content 0 7 mg/ml) were obtained from Behringwerke AG Marburg/Lahn, Germany

HCS estimation Serum levels of HCS were determined by radio-immuno-assay as described by *Lebech & Borggaard* (20)

RESULTS

In Fig 1 the percentage of B lymphocytes found at certain stages during pregnancy is compared with the percentage in non-pregnant controls and the results obtained in an earlier study of 16 women during a period ranging from 4 to 203 hours after delivery (9) The difference between the percentages in pregnant women at 33–40 week of gestation and those in the controls is statistically significant, $0.020 < p < 0.025$ There is no difference in B cell percentage in the women immediately before and after delivery

Table 1 shows the percentual distribution of the B T and null cells. No change in the T cell percentage is seen during pregnancy Consequently the diminution of B lymphocytes is reflected in a raised null cell percentage

Table 2 shows the results in absolute number A decline in the mean values for B lymphocytes is noted although the difference is not statistically significant.

Table 3 shows the serum concentrations of IgG IgM IgA, C4 and C1IA. No obvious

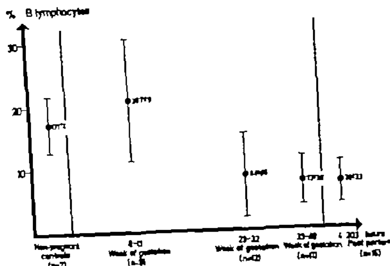


Fig. 1 The mean values of B cell percentage (± 1 S.D.) at various stages during pregnancy shown together with the values in non-pregnant controls and the post-partum group.

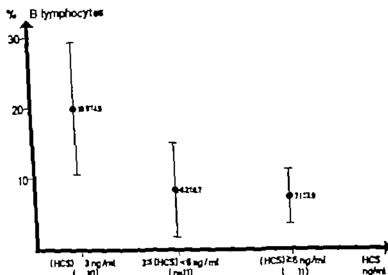


Fig. 2 The mean values of the B cell percentage, within various ranges of HCS concentration, in the pregnant women (± 1 S.D.)

changes in these parameters during pregnancy are found.

In Fig. 2, the B cell percentage is related to the HCS concentrations in serum. It is seen that there is an inverse relationship between the B cell percentage and the HCS concentration.

DISCUSSION

The present study reveals a significant percentual diminution of the B lymphocytes during pregnancy. This is accompanied by a decline in absolute number of B cells, although this is not statistically significant. No changes in the T lymphocyte percentage or absolute

TABLE 1 *The Percentage of B, T and Null Lymphocytes at Certain Stages During Pregnancy*

	B cells	T cells	Null cells
Controls (7)	17.1 ± 4.5	58.1 ± 6.2	24.7 ± 7.8
8–11 week (9)	20.7 ± 9.7	56.6 ± 3.3	[illegible]2.7 ± 12.2
23–32 week (12)	8.4 ± 6.6	57.2 ± 5.2	34.4 ± 9.0
33–40 week (11)	7.3 ± 3.8*	60.0 ± 7.2	32.7 ± 9.6

* Significantly different from the controls 0.0[illegible]0<p<0.025

TABLE 2 *Distribution of Leucocytes and Lymphocyte Subpopulations at Certain Stages During Pregnancy*

	Leucocytes	Lymphocytes	B cells	T cells	Null cells
Controls (7)	6000 ± 1070	1925 ± 560	325 ± 120	1103 ± 270	475 ± 270
8–11 week (9)	7500 ± 2700	1934 ± 710	387 ± 189	108[illegible] ± 380	480 ± 390
23–32 week (12)	10700 ± 3200	2362 ± 370	208 ± 171	1361 ± 250	809 ± 202
33–40 week (11)	9720 ± 4110	2051 ± 1130	144 ± 102	1251 ± 621	791 ± 610

Absolute number per µl ± 1 S.D

TABLE 3 *Serum Concentrations of Immunoglobulins, C4 and C1 Inactivator at Certain Stages During Pregnancy*

	IgG	IgM	IgA	C4	C1IA
Controls (7)	960 ± 355	75 ± 23	210 ± 119	38 ± 6	40 ± 4
8–11 week (9)	897 ± 256	77 ± 54	164 ± 84	45 ± 12	34 ± 11
23–32 week (12)	837 ± 155	83 ± 38	189 ± 95	43 ± 14	3[illegible] ± 6
33–40 week (11)	704 ± 181	90 ± 68	172 ± 60	53 ± 2[illegible]	31 ± 6

Concentrations expressed in mg per 100 ml ± 1 S.D

number were found. Consequently a rise in amount of null cells was seen.

To our knowledge, only a few papers on the quantitative distribution of the lymphocyte subpopulations during pregnancy are available.

Gergely et al. (16) found a slight increase in the B cell percentage and number, whereas *Brain et al.* (6) found no significant change.

The discrepancy between our results and those obtained in the two studies referred to, might be due to the fact that we have used a cytophotometric measurement of the fluorescence. The decline in the B cell percentage corresponds well to our earlier findings in a series of 16 women shortly after the termination of their pregnancy (9). It is, however, not possible to establish whether our results are due to a true decline in number of B cells or they reflect a blocking effect of some components present in blood which during pregnancy interfere with the detection system.

As regards T lymphocytes, our results are in accordance with the results obtained by *Campion & Currey* (8) and *Gergely et al.* (16), who found no quantitative change of the T lymphocytes during pregnancy. Hence it seems reasonable to suggest that the reduced PHA response of lymphocytes in preg-

nant women and lymphocytes incubated with plasma from pregnant women, is not a consequence of a decline in the actual number of T lymphocytes.

Leikin (21) found that a 20 per cent solution of plasma obtained from mothers at the time of parturition was suppressive *in vitro* to cells from pregnant and non-pregnant women and suggested that the depressed lymphocyte response is due to a plasma factor and not to intrinsic cellular changes.

In order to see whether this plasma factor might be a part of the normal humoral defence system, serum concentrations of immunoglobulins, C4 and C1IA were investigated. The concentration of C1IA is altered in certain pathological conditions, e.g. raised in some neoplastic conditions, possibly blocking the humoral immune reaction (2). Our results, however, do not give evidence for a similar hypothesis according to which a blocking of the complement system at the C1 level may be involved in the protection of the foetus.

Human Chorionic Gonadotropin (HCG) suppression of the reactivity in mixed lymphocyte cultures has been reported by *Beling & Weksler* (4) and several workers have shown an inhibitory effect *in vitro* of HCG on lymphocyte blastogenic response to PHA (1, 17).

Investigations by *Contractor & Davies* (10) have demonstrated that "*in vitro*" incubation of male donor lymphocytes with HCG or HCS in physiological concentrations causes a reduced response on PHA stimulation.

As it is well-established that the serum concentration of HCG declines after the first trimester whereas HCS rises throughout the period of pregnancy we have only related the percentage of B lymphocytes to the serum concentration of HCS (Fig. 2). It is seen that the decline in B cells is concomitant to the rise in HCS concentration.

We suggest that the depression of the B lymphocytes might be of importance for the survival of the foetus. Human Chorionic Somatomammotropin might be one of the serum factors responsible for the alteration in lymphocyte reactivity during a normal pregnancy.

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REFERENCES

1. *Adcock, E. W., Teasdale, F., August, C. S., Cox, S., Meschia, G., Battaglia, F. C. & Naughton, M. A.:* Human Chorionic Gonadotropin: Its possible role in maternal lymphocyte suppression. Science *181*, 845–847, 1973.
2. *Bach-Mortensen, N., Osther, K. & Strøyer, I.:* C1-esterase Inactivators and C4 in malignant diseases. Lancet *ii*, 499–500, 1975.
3. *Beer, A. E. & Billingham, R. E.:* The embryo as a transplant. Scientific American. *230*, 36–46, 1974.
4. *Beling, C. G. & Weksler, M. E.:* Suppression of mixed lymphocyte reactivity by human chorionic gonadotropin. Clin. exp. Immunol. *18*, 537–541, 1974.
5. *Brain, P. & Gordon, J.:* Rosette formation by peripheral lymphocytes II. Inhibition of the phenomenon. Clin. exp. Immunol. *8*, 441–444, 1971.
6. *Brain, P., Marston, R. H. & Gordon, J.:* Immunological responses in pregnancy. Brit. Med. J. *4*, 488, 1972.
7. *Bøyum, A. V.:* Separations of leucocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. Suppl. *97*, 51–76, 1968.
8. *Campion, P. D. & Currey, H. L. F.:* Cell-mediated immunity in pregnancy. Lancet *ii*, 830, 1972.
9. *Christiansen, J. S., Osther, K., Pedersen, B. & Bach-Mortensen, N.:* B, T and null lymphocytes in newborn infants and their mothers. Acta Ped. Scand. In press.
10. *Contractor, S. F. & Davies, H.:* Effect of human chorionic somatomammotrophin and human chorionic gonadotrophin on phytohaemagglutinin-induced lymphocyte transformation. Nature. New Biol. *243*, 284–286, 1973.
11. *Finn, R., St. Hill, C. A., Govan, A. J., Ralfs, I. G., Gurney, F. J. & Denye, V.:* Immunological responses in pregnancy and survival of foetal homograft. Brit. Med. J. *3*, 150–152, 1972.
12. *Fothergill, J. E.:* Properties of conjugated serum proteins. I: R. C. Nairn, (Ed.): *Fluorescent protein tracing*. Livingstone, Edinburgh & London 1969, p. 35–60.
13. *Frøland, S. S. & Natvig, J. B.:* Surface-bound immunoglobulin on lymphocytes from normal

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23–32 week (12)	8 4 ± 6 6	57.2 ± 5.2	34 4 ± 9.0
33–40 week (11)	7.3 ± 3 8*	60 0 ± 7.2	32.7 ± 9.6

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ALLO-AGGRESSION IN CHICKENS

II. Cellular Expression of the AA Gene Products

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Crone, M. & Simonsen, M. Allo-aggression in chickens. II. Cellular expression of the AA gene products. Acta path. microbiol. scand. Sect. C, 84: 319–324, 1976.

B1-anti-B2 and B2-anti-B1 allo-antisera were raised by i.v. immunization of homozygous $B^{1/1}$ and $B^{2/2}$ chickens, respectively, with $B^{2/1}$ cells of the following sources: peripheral blood leucocytes (PBL), erythrocytes, thymus cells and bursa cells. Purified suspensions of these all produced anti-B allo-antibodies which could both haemagglutinate red blood cells and strongly inhibit the GVH reactivity of blood lymphocytes of the original donor type. Absorption studies of the sera using cells of the same four sources showed that they could all completely remove the GVH inhibitory antibodies. A fact of particular importance is that B-haplotype determined antigens which are common to erythrocytes and lymphocytes seem to include the gene products of the allo-aggression locus, as earlier defined (*Simonsen* 1975).

Key words: Allo-aggression; AA gene products; cellular expression; chickens.

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The major histocompatibility complex (MHC) in the chicken, the B-complex, has histocompatibility effects comparable with those of the H-2 and HL-A systems in mice and man. It was first described by *Briles et al.* (1950) as a blood group locus and later *Schierman & Nordskog* (1961) also identified it as the major skin graft locus. Subsequently the major locus controlling GVH splenomegaly was also found to form part of the B-complex (*Jaffe & McDermid* 1962, *Simonsen* 1975) and the same applies to the recently described mixed lymphocyte reaction (MLC) locus (*Miggiano et al.* 1974).

It was suggested already by *Schierman & Nordskog* (1962) that some B antigens may be common to lymphocytes and erythrocytes. In tolerance experiments where they injected 1-day-old chickens with allogeneic erythrocytes, the production of agglutinins for lymphocytes as well as for erythrocytes was inhibited if subsequent immunization was attempted. In haemagglutination- and absorption experiments, B-complex antigens have been detected also on fibroblasts (*Hložánek & Hála* 1969), on bone marrow and liver cells (*Benda & Hála* 1972) and on thymocytes (*Wong* 1974).

It has been shown that GVH splenomegaly in chicken embryos can be quantitatively suppressed by allo-antibodies directed against certain B-complex antigens of the donor cells (*McBride et al.* 1966, *Crone et al.* 1972). However, many of the haemagglutinating antibody specificities raised by a given B-haplotype difference are apparently irrelevant in GVH inhibition and can be removed by ab-

and immunodeficient humans. Scand J Immunol *1* 1–12 1972

Frøland S S Binding of sheep erythrocytes to human lymphocytes. A probable marker of T-lymphocytes. Scand J Immunol. *1* 269–280 1972

Gatti R A Yunis E J & Good R A Characterization of a serum inhibitor of MLC reactions. Clin. exp Immunol *13* 427–437 1973

. *Gergely P Dzvonyar J Szegedi Gy Fekete B Szabo G & Petranyi Gy* T and B lymphocytes in pregnancy Klin. Wschr *52* 601–602 1974

Han T Inhibitory effect of human chorionic gonadotrophin on lymphocyte blastogenic response to mitogen antigen and allogenic cells. Clin exp Immunol *18* 529–537 1974

. *Kasakura S* A factor in maternal plasma during pregnancy that suppresses the reactivity of mixed leucocyte cultures. J Immunol *107* 1296–1301 1971

Laurell, C B Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Analyt. Biochem. *15* 45–52 1966.

Lebech P E & Borggaard B Serum levels of Human Chorionic Somatomammotrophin (HCS) in normal and abnormal pregnancies. Acta Endocrinol. Suppl. *182* 35–43 1973

Leikin S Depressed maximal lymphocyte response to phytohaemagglutinin in pregnancy Lancet ii 43 1972

22 *Osther K & Dybkjaer E.* Complement components on the surface of normal human lymphocytes. Scand. J Haematol. *13* 24–32, 1974

23 *Purtilo D T Hallgren H M & Yunis E J* Depressed maternal lymphocyte response to phytohaemagglutinin in human pregnancy Lancet *i* 769–771 1972

24 *Rabellino E & Grey H M.* Immunoglobulins on the surface of lymphocytes III Surface Immunoglobulins in chicken lymphocytes J Immunol *106* 1418–1420 1971

25 *St Hill C A., Finn R & Denye V.* Depression of cellular immunity in pregnancy due to a serum factor Brit. Med. J *3* 513–514 1973

26 *Stimson W H* Transplantation—nature's success. Lancet *i* 684 1972.

27 *Unanue E R Grey H M Rabellino E, Campbell P & Schmidtke J* Immunoglobulins on the surface of lymphocytes II J Exp. Med. *133* 1188–1198, 1971

28. *Walker J S Freeman C B & Harris, R.* Lymphocyte reactivity in pregnancy Brit. Med. J *3* 469 1972.

29 *Wybran J Carr M D & Fudenberg, H H* The human rosette-forming cells as a marker of a population of thymus-derived cells. J. clin. Invest *51* 2537–2543 1972.

30 *Yu V Y H Walker C A MacLennan I C M & Beum J D* Lymphocyte reactivity in pregnant women and newborn infants. Brit. Med. J *1* 428 1975

RESULTS

Four different batches of allo-antisera have been examined. Two batches were produced in B^{r} chickens immunized with B^{t} PBL and erythrocytes, respectively These were produced simultaneously using cells from the same donor panel and were absorbed and tested in GVH assays together Each batch was a pool from three animals.

Titration of the GVH inhibitory potency of these sera is shown in Table 1 The anti-B1 sera diluted 1.2,000 give maximum inhibition while the dilutions of 1.50,000 show no inhibitory effect. A control serum produced by immunization with low doses of erythrocytes corresponding to the number of erythrocytes contaminating the lymphocyte suspensions used for the production of antiserum I is not shown in the table. It gave no GVH inhibition, not even in a dilution of 1.20 and it did not haemagglutinate either

These two allo-antisera (I & II) had haemagglutinating antibodies against B^{4} erythrocytes in titres of 2 to 2^{4} This shows, as also pointed out earlier (*Crone* 1973) that inhibition of GVH reactivity is a test for allo-antibodies that is much more sensitive than haemagglutination.

The two anti-B1 allo-antisera were then absorbed in dilutions of 1.2,000 with graded numbers of $B^{r/}$ PBL and erythrocytes in order to see whether the GVH inhibition

TABLE 1 *Inhibition of GVH Activity of B4 Lymphocytes with Anti-B1 Allo-antisera I and II Raised with PBL and Erythrocytes Respectively*

Dilution of allo-antiserum during incubation	I anti-B1 lymphocyte	II anti-B1 erythrocyte
1 2,000	1.21±0 03 (7)	1 15±0 03 (7)
1 10,000	1 46±0 03 (8)	1 37±0.06 (8)
1 50,000	1 73±0 05 (7)	n.d.
N antiserum	1 74±0 06 (7)	
No cells	1 13±0 03 (11)	

Spleen weights expressed as mean log spleen weight (mg) ± S E. Numbers in bracket are numbers of embryos per group

TABLE 2. *Inhibition of GVH Activity of B Lymphocytes Using Allo-antisera I and II Absorbed with B4/1 PBL and Erythrocytes*

No. of B^{H} cells used to absorb 1 ml antiserum 1:2,000	I anti-B1 lymphocyte	II anti-B1 erythrocyte
1.5×10⁶ ly	1.53±0 06 (8)	1.37±0 08 (9)
1.5×10⁷ ly	1.90±0.09 (8)	1 77±0 05 (9)
1.5×10⁸ ly	1.99±0 04 (7)	1.97±0 08 (9)
1.5×10⁶ erythr	1 43±0.06 (8)	1.54±0 08 (9)
1.5×10⁷ erythr	1.88±0 11 (8)	1.67±0 04 (9)
1.5×10⁸ erythr	2.21±0.06 (8)	1.86±0 07 (9)
Controls		
No antiserum	2.14±0 09 (6)	1 80±0.04 (9)
Unabsorbed antiserum	1.59±0.05 (8)	1 40±0 05 (9)

Spleen weights expressed as mean log spleen weight (mg) ± S E. Numbers in brackets are numbers of embryos per group.

could be quantitatively removed by both cell types.

Table 2 shows that doses of 1.5 × 10⁸ of either cell type removed the GVH inhibitory antibodies totally from both antisera. Also lower doses absorbed equally well.

Extensive control absorptions of these sera were performed with B^{r} cells (not shown) but a hint of non-specific removal of the anti-B1 antibodies was not seen in any case.

Allo-antiserum III (anti-B2-bursa serum) was raised by immunization of a B^{1} chicken with B^{r} bursa cells. The haemagglutination titre was 2^{4} and strong inhibition of GVH activity was seen after incubation of B^{r} cells in a final antiserum concentration 1.300 (incubation of equal vol. of cells and antiserum 1 150) A final concentration of 1 1,000 was partially inhibitory while 1.5,000 had no effect. Samples of the serum dilution 1 150 were then absorbed and tested the results are shown in Table 3

It is seen that absorption with B^{r} erythrocytes, bursa cells and thymus cells removed the inhibitory antibodies equally well. Control absorptions with $B^{r/}$ cells had no effect.

Allo-antiserum IV (anti-B1 thymus serum) was produced in a B^{r} chicken after only 4

sorption with unrelated blood cells which cannot remove the GVH inhibitory anti bodies. By such absorptions, *Simonsen* (1975) produced antisera which appeared to be spe cific for the gene product(s) responsible for the participation of the donor cells in the GVH reaction. Such sera could prevent the GVH reaction by their action on the donor lymphocytes and yet they retained some of their haemagglutinating effect on the donor erythrocytes. These sera could also be used for sero-typing of unrelated birds, a typing which permitted the selection of donors com patible with a given recipient in terms of allo-aggression (AA) as measured by GVH splenomegaly The term of AA locus was suggested for the pertinent part of the B-haplotype.

The main purpose of the present work was to investigate whether chicken erythrocytes actually express the AA gene products in the membrane. As a corollary also thymic and bursal lymphocytes were investigated together with PBL. These four cell sources were employed both for stimulating the production of antibodies and for absorbing the antisera produced The main conclusion is that the AA gene products are indeed present on ery throcytes as well as on lymphoid cells.

MATERIAL AND METHODS

Animals Partly inbred lines of homozygous $B^{1/1}$ and $B^{2/2}$ chickens and their F1 hybrids were used for the production of allo-antisera and as cell donors for the GVH assays. These lines were originally supplied by Drs. *L. W. Schierman* and *R. A. McBride* and have been kept by random matings within the lines. Fertilized eggs from out bred White Leghorns obtained from a commercial hatchery were used as recipients in the GVH assays

Allo-antisera. The immunization schedule for preparation of the allo-antisera included weekly intravenous injection of 1–5 × 10^6 $B^{2/2}$ cells into homozygous $B^{1/1}$ (anti-B2 serum) or homozygous $B^{2/2}$ (anti B1 serum) The animals were bled after 5 to 7 immunizations on day 7 after the last injection, unless otherwise stated. Different batches of allo-antisera were produced in response to the following cells

(1) *PBL* recovered from heparinized blood after light centrifugation The contamination with erythrocytes was less than 1 per cent.

(2) *Purified erythrocytes* obtained after removal of the leucocytes, followed by three to five washings in phosphate buffered saline (PBS) pH 7.3 The contaminating leucocytes in the upper layer were removed after each centrifugation. The final erythrocyte suspension contained less than 0.01 per cent leucocytes.

(3) *Bursa cells* obtained from 5–6 weeks old chickens. At this age, the chicken bursa have very few T cells estimated by immunofluorescence with rabbit anti chicken thymus serum made specific by absorption with bursa cells (M.C. unpublished) Single cell suspensions were prepared by homogenization through a metal grid followed by filtration through gauze Contamination with erythrocytes was less than 1 per cent.

(4) *Thymus cells* were obtained from *bursectomized* chickens 6–8 weeks old in order to avoid the B cells (2–5 per cent) usually present in normal chicken thymus. Bursectomy was performed as earlier described (*Jensenius et al.* 1975) Only chickens with less than 0.01 mg/ml of serum Ig (as estimated by rocket immunoelectrophoresis) were used as thymus donors. Single cell suspensions were obtained in the same way as bursa cell suspensions. The contamination with erythrocytes was less than 0.5 per cent

Inhibition of GVH by allo-antisera was per formed as earlier described (*Crone* 1973) Briefly $B^{1/1}$ or $B^{2/2}$ PBL were incubated for one hour at 37 C with appropriate dilutions of anti-B1 or anti B2 allo-antiserum, respectively (for details see tables) A standard volume of 0.10 ml of the incubation mixture containing 1.5 × 10^6 leucocytes was injected i.v. into groups of 13–14-day-old outbred White Leghorn embryos which were killed for removal of the spleen 6 days later The mean log spleen weight in each group (6–10 embryos) was calculated.

Absorption of allo-antisera. The different anti-B1 allo-antisera were first titrated in PBS for their inhibitory effect on the GVH reactivity of $B^{1/1}$ lymphocytes. Samples of the lowest concentration of antiserum giving almost complete inhibition were prepared in PBS containing 2 per cent BSA (Sigma, Cohn's fraction V) and were absorbed with test cells from $B^{1/1}$ animals and control cells from $B^{2/2}$ animals Suspensions of PBL, erythrocytes, bursa cells and thymus cells were used for absorption. Their preparation and contamination with other cells were as mentioned above under production of allo-antisera The suspensions were washed three times in medium with 2 per cent BSA and cell pellets after the last centrifugation were resuspended in the diluted antiserum and left to absorb for 1 hour at 4 C The absorbed sera were millipore filtered quickly frozen, and stored at −20 C.

Haemagglutination titres were determined by Takatsy plates using 2 fold dilutions of sera in PBS and 2 per cent erythrocyte suspensions, also in PBS.

RESULTS

Four different batches of allo-antisera have been examined. Two batches were produced in B^{4} chickens immunized with B^{14} PBL and erythrocytes, respectively. These were produced simultaneously using cells from the same donor panel and were absorbed and tested in GVH assays together. Each batch was a pool from three animals.

Titration of the GVH inhibitory potency of these sera is shown in Table 1. The anti-B1 sera diluted 1:2,000 give maximum inhibition while the dilutions of 1:50,000 show no inhibitory effect. A control serum produced by immunization with low doses of erythrocytes corresponding to the number of erythrocytes contaminating the lymphocyte suspensions used for the production of antiserum I is not shown in the table. It gave no GVH inhibition, not even in a dilution of 1:20, and it did not haemagglutinate either.

These two allo-antisera (I & II) had haemagglutinating antibodies against B^{11} erythrocytes in titres of 2^{3} to 2^{6}. This shows, as also pointed out earlier (*Crone* 1973), that inhibition of GVH reactivity is a test for allo-antibodies that is much more sensitive than haemagglutination.

The two anti-B1 allo-antisera were then absorbed in dilutions of 1:2,000 with graded numbers of B^{11} PBL and erythrocytes in order to see whether the GVH inhibition could be quantitatively removed by both cell types.

Table 2 shows that doses of 1.5×10^{8} of either cell type removed the GVH inhibitory antibodies totally from both antisera. Also lower doses absorbed equally well.

Extensive control absorptions of these sera were performed with B^{4} cells (not shown), but a hint of non-specific removal of the anti-B1 antibodies was not seen in any case.

Allo-antiserum III (anti-B2-bursa serum) was raised by immunization of a B^{1} chicken with B^{2} bursa cells. The haemagglutination titre was 2^{4} and strong inhibition of GVH activity was seen after incubation of B^{2} cells in a final antiserum concentration 1:300 (incubation of equal vol. of cells and antiserum 1:150). A final concentration of 1:1,000 was partially inhibitory, while 1:5,000 had no effect. Samples of the serum dilution 1:150 were then absorbed and tested; the results are shown in Table 3.

It is seen that absorption with $B^{2/2}$ erythrocytes, bursa cells and thymus cells removed the inhibitory antibodies equally well. Control absorptions with $B^{1/1}$ cells had no effect.

Allo-antiserum IV (anti-B1-thymus serum) was produced in a B^{4} chicken after only 4

TABLE 1. *Inhibition of GVH Activity of B^{14} Lymphocytes with Anti-B1 Allo-antisera I and II Raised with PBL and Erythrocytes, Respectively*

Dilution of allo-antiserum during incubation	I anti-B1 lymphocyte	II anti-B1 erythrocyte
1:2,000	1.21 ± 0.03 (7)	1.15 ± 0.03 (7)
1:10,000	1.46 ± 0.05 (8)	1.37 ± 0.06 (8)
1:50,000	1.73 ± 0.05 (7)	d.
No antiserum	1.74 ± 0.06 (7)	
No cells	1.13 ± 0.03 (11)	

Spleen weights expressed as mean log spleen weight (mg) ± S.E. Numbers in brackets are numbers of embryos per group.

TABLE 2. *Inhibition of GVH Activity of B^{1} Lymphocytes Using Allo-antisera I and II Absorbed with B^{11} PBL and Erythrocytes*

No. of B^{11} cells used to absorb 1 ml antiserum 1:2,000	I anti-B1 lymphocyte	II anti-B1 erythrocyte
1.5×10^{6} ly	1.53 ± 0.06 (8)	1.37 ± 0.08 (9)
1.5×10^{7} ly	1.90 ± 0.09 (8)	1.77 ± 0.05 (9)
1.5×10^{8} ly	1.99 ± 0.04 (7)	1.97 ± 0.08 (9)
1.5×10^{6} erythr	1.43 ± 0.06 (8)	1.54 ± 0.08 (9)
1.5×10^{7} erythr	1.88 ± 0.11 (8)	1.67 ± 0.04 (9)
1.5×10^{8} erythr	2.21 ± 0.06 (8)	1.86 ± 0.07 (9)
Controls.		
No antiserum	2.14 ± 0.09 (6)	1.80 ± 0.04 (9)
Unabsorbed antiserum	1.39 ± 0.05 (8)	1.40 ± 0.05 (9)

Spleen weights expressed as mean log spleen weight (mg) ± S.E. Numbers in brackets are numbers of embryos per group.

TABLE 3 *Inhibition of GVH Activity of* $B^{2/2}$ *Lymphocytes Using an Allo-antiserum Raised with* $B^{2/2}$ *Bursa Cells*

Cells used for absorption of 1 ml of allo-antiserum 1 150	Mean log spleen weight ± S E
10^7 $B^{2/2}$ erythrocytes	1 29±0 07 (5)
10^8	1 77±0.07 (4)
10^9 "	1 70±0 06 (7)
10^7 $B^{2/2}$ thymus cells	1.50±0 11 (4)
10^8 "	1 65±0 06 (6)
10^9 "	1 68±0 08 (6)
10^7 $B^{2/2}$ bursa cells	1.39±0 12 (6)
10^8 "	1 89±0 08 (6)
10^9 "	1 66±0 10 (3)
10^9 $B^{1/1}$ erythrocytes	1.38±0 09 (3)
10^9 $B^{1/1}$ thymus cells	1 28±0 09 (5)
10^9 $B^{1/1}$ bursa cells	1 37±0 11 (2)
Cell control (no antiserum)	1 87±0 02 (5)
Unabsorbed antiserum	1.39±0 08 (6)

Spleen weights expressed as mean log spleen weight (mg) ± S E Numbers in brackets are numbers of embryos per group

immunizations with thymus cells from bursectomized chickens containing the B^1 haplotype. The haemagglutination titre was 2^4 and an almost complete inhibition of GVH activity of $B^{1/1}$ leucocytes was found when the cells were pretreated with the antiserum in a final concentration of 1 200 (equal amounts of cells and antiserum 1 100) Inhibition was not found after incubation in antiserum 1 1 000 Data from absorption of the antiserum 1 100 using different doses of $B^{1/1}$ erythrocytes, thymus cells and bursa cells are shown in Table 4 Table 4 shows also that all cell types could remove the inhibitory antibodies Bursa cells and erythrocytes were just as efficient as thymus cells. Control absorptions using $B^{2/2}$ thymus cells, erythrocytes and bursa cells had no effect (not shown)

Although the data recorded in Table 4 gave no hint of the presence of thymus cell specific antibodies it could be argued that they might have escaped detection because they were diluted out in the 1 100 serum dilution employed for absorptions. A new absorption experiment was therefore performed using the same antiserum in the dilution 1 10. Five consecutive absorptions with $B^{1/1}$ erythrocytes were carried out and 5 control absorptions with $B^{2/2}$ erythrocytes. Samples were taken from each absorption and tested simultaneously in GVH inhibition. Also these data showed complete removal of GVH inhibition with $B^{1/1}$ erythrocytes, but no demonstrable diminution even after 5 control absorptions.

DISCUSSION

At first sight it may seem to be of minor importance whether an MHC-determined antigen known to be expressed in T lymphocytes happens to be present also in erythrocytes. Classical examples of such dual expression are the K and D locus antigens of the H 2 chromosome in mice, while no HL-A specificities in man seem to be demonstrable on mature erythrocytes. Even so there are reasons why we have taken much interest in this particular point in the case of the chicken MHC. Thus, it has actually been possible to develop a typing system where avoidance of

TABLE 4 *Inhibition of GVH Activity of* $B^{1/1}$ *Lymphocytes Using an Allo-antiserum Raised with Thymus Cells from Bursectomized Donors Containing the B1 Haplotype*

Cells used for absorption of 1 ml of allo-antiserum 1 100	Mean log spleen weight ± S.E.
10 $B^{1/1}$ erythrocytes	1 19±0 10 (4)
10	1 46±0 09 (4)
10^9	1.50±0 08 (6)
10^7 B / thymus cells	1.20±0.07 (6)
10^8	1.35±0 13 (3)
10^9	1.50±0 05 (4)
10 B /1 bursa cells	1 19±0 02 (3)
10^8	1.54±0 06 (6)
10^9	1 62±0 08 (4)
Cell control (no antiserum)	1 45±0 05 (3)
Unabsorbed antiserum	1.20±0.05 (7)

Spleen weights expressed as mean log spleen weight (mg) ± S E. Numbers in brackets are numbers of embryos per group

allo-aggression is based on a haemagglutination test (*Simonsen* 1973 1975) Although the most simple explanation of this fact assumes that erythrocytes and T lymphocytes share an important MHC antigen, the alternative is 2 or more firmly linked, though quite different genes, one of which is expressed in erythrocytes and the other(s) in T cells. The present demonstration of the fact that GVH-inhibitory antibodies can be raised as well as removed by purified red blood cells makes this alternative explanation untenable.

However although the present data are in support of the assumption of a shared antigen in erythrocytes and T cells of crucial importance for allo-aggression, they leave the genetic basis of that phenotypic association quite unsettled. At least 2 main possibilities have to be considered. (a) A single locus may be phenotypically expressed in both cell types (as well as in bursa cells and probably in other cell types too) (b) All members of a multigene family (gene cluster) of firmly linked and similar though not identical, genes (*Silver & Hood* 1976) may specify common framework determinants which constitute the AA antigens. This may be subdivided into three possibilities whether the AA-bearing molecules are seen in the role of stimulators or in the role of receptors in allo-aggression, or both.

If (b) is correct, there is no evidence at hand to suggest that the same number of genes are expressed in the erythrocyte and T cell populations of a given individual. In fact, if only one gene of the family" were to be expressed in erythrocytes though they are all expressed in T cells, it would explain the immunization and absorption data just as well, since both cell types would still be sharing identical AA antigens.

We prefer possibility (b) rather than (a) mainly because the former is by far more helpful in attempts to explain T cell recognition in terms of gene products of the MHC (*Crone et al.* 1972 and *Simonsen* 1976) by now however this is no more than a clearly stated bias.

Our inability to find GVH-inhibitory thymus-specific allo-antibodies can best be ascribed to bad luck, since 2 other groups by now have discovered such antibodies by way of reciprocal thymus cell injections into 2 inbred lines of the same MHC type. Presumably the 2 reports are concerned with the same locus which has been named Th1 (*Gilmour* 1975) and Ly4 (*Fredericksen et al.* 1975) respectively Dr *Gilmour* has kindly provided us with typing sera to the 2 alleles he has so far defined, Th-1 1 and Th-1.2 and so far we have found our inbred B^1 and B^4 birds to react only with the former (unpublished) Hence, it seems reasonable that we have not been able to see antibodies ascribable to a Th-locus difference in the data recorded in Table 4

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REFERENCES

1 *Benda V & Hála K* Relative distribution of B antigens in erythrocytes and some other chicken tissues. Folia Biol. (Praha) *18* 301 304 1972.
2. *Briles W E., McGibbon, W H & Irwin M R.* On multiple alleles effecting cellular antigens in the chicken. Genetics *35* 633–652 1950.
3 *Crone M* Tolerance to allogeneic cells induced in bursectomized chickens. Scand. J Immunol. 2 349–355, 1973
4 *Crone M Koch, C. & Simonsen, M.:* The elusive T cell receptor Transplant. Rev *10* 36–56, 1972.
5 *Fredericksen T Longenecker B M., Pazderka F., Stone H A. & Ruth, R. F* A fourth lymphocyte antigen system in the chicken demonstrated by inhibition of GVH splenomegaly Fed Proc. *34* 1033 1975
6 *Gilmour D G* Two loci determining alloantigens of bursal or thymic cells in the chicken Folia Biol (Praha) *21* 359 1975
7 *Hlaváček I & Hála, K.* The detection of B red blood cell allele on chick embryo fibroblasts. Folia Biol. (Praha) *15* 474–477 1969
8 *Jaff W P & McDermid E. M* Blood groups and splenomegaly in chick embryos. Science *137* 984–984 1962.

Jensenius J C Crone M & Koch C Antigen binding by non-bursa-derived chicken leukocytes. Scand. J Immunol. *4* 151–160 1975

McBride R A., Coppleson L. W Nisbet N W Simonsen M Skowron-Cendrzak A & Wigzell H L. R Accelerated immunological maturation in the chick. Immunology *10* 63–79 1966

Miggiano I C., Birgen I & Pink J R L. The mixed lymphocyte reaction in chickens. Evidence for control by the major histocompatibility complex. Eur J Immunol. *4* 397–401 1974

. *Schierman L. W & Nordskog A W* Relationship of blood type to histocompatibility in chickens. Science *134* 1008–1009 1961

Schierman L. W & Nordskog A W Relationship of erythrocyte to leucocyte antigens in chickens. Science *137* 620–621 1962.

Silver J & Hood L. Preliminary amino acid sequences of transplantation antigens Genetic and evolutionary implications. Cont Topics Molec. Immunol In press.

15 *Simonsen M* Tissue Typing An experimental solution in the chicken. J Immunol. *111* 1607–1609 1973

16 *Simonsen M.* Allo-aggression in chickens Serological identification of the major genetic locus. Acta path. microbiol scand. Sect. C, *83* 1–14 1975

17 *Simonsen M* In Katz, D H. and Benacerraf B (Eds.) The role of products of the histocompatibility gene complex in immune responses. Academic Press New York 1976. p. 752.

18. *Wong S Y* Adherence of chicken lymphocytes to surfaces. Ph.D Thesis 1974 Dep. of Zoology University of Alberta, Edmonton, Canada. (Cited from *Pazderka et al* The Major Histocompatibility Complex of the Chicken. Immunogenetics 2 *101–130 1975*)

Acta path. microbiol. scand. Sect. C, 84: 325–332, 1976

SYNERGISTIC EFFECT OF ALLOANTIBODIES OR $F(ab')_2$ AND PREDNISONE ON MURINE SPLIT HEART ALLOGRAFT SURVIVAL

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Schilling, W. & Svehag, S.-E. Synergistic effect of alloantibodies or $F(ab')_2$ and prednisone on murine split heart allograft survival. Acta path. microbiol. scand. Sect. C, 84: 325–332, 1976.

Heterotopic grafting of split allogeneic mouse hearts across a strong H-2 barrier (A/Sn → ACA) was used in a study of alloantibody or $F(ab')_2$ induced enhancement. Strong specific binding of alloantibodies occurred in the graft. A marked synergistic effect on graft survival was observed after combined treatment of recipients with enhancing serum and prednisone. The highly dose-dependent enhancing effect of $F(ab')_2$ was also strongly potentiated by supporting prednisone treatment. *In vitro* pre-incubation of the heart tissue with alloantiserum or $F(ab')_2$ did not prolong graft survival. In contrast, C-mediated damage of the graft was suggested when serum was used.

Key words: Passive enhancement; alloantibody; $F(ab')_2$; prednisone.

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The shortcomings of non-specific immunosuppression in clinical transplantation are of such magnitude that continued efforts to define clinically applicable methods of specific immunosuppression are necessary. Immunological enhancement in rodents (1, 4, 6–10, 14) represents a useful model for studies of donor-specific immunosuppression. Alloantibodies that mediate enhancement are directed against gene products of the major histocompatibility genetic complex (MHC). However, both experimental and clinical findings (4, 6, 9, 12, 15, 16) indicate that alloantibodies can damage organ transplants by complement mediated cytotoxicity reactions. In attempts to induce passive enhancement it should be possible to eliminate this risk of damage to the graft by the use of non-complement factor 1 fixing Fab or $F(ab')_2$ fragments (7).

We have employed a cardiac allograft model in mice (20) to study the influence of factors such as dosage, treatment schedule and synergism with prednisone in primarily $F(ab')_2$-induced enhancement.

MATERIALS AND METHODS

Cardiac Transplantation

A technique earlier described (20) was used for heterotopic grafting of split allogeneic mouse

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hearts. Donor hearts excised from 12 to 36 h old baby mice, were sectioned into two parts along the ventricular septum. Each half of a heart was inserted into a 3 mm deep s.c. pouch on the dorsal pinna of a recipient. No sutures were applied. The electrical activity of the transplants was monitored by a Tektronic 410 cardioscope, starting on day 7 after transplantation. The per cent graft takes varied from 80 to 100 per cent in different experiments.

Alloantiserum

Alloantiserum was prepared by injecting A.CA mice with 0.1 ml A/Sn spleen cell suspension (5×10^6 cells) plus 0.05 ml Freund's incomplete adjuvant i.p. every 10th day. On one occasion, an A/Sn heart cell suspension (3×10^6 cells) was used for immunization. Antisera were collected, starting 2 months after the initial antigen injection.

Cytotoxic Antibody Assay

Cytotoxic antibodies were assayed by a radiochromium technique (21). Heat-inactivated alloantiserum or $F(ab')_2$ solutions (0.075 ml) and ^{51}Cr-labelled A/Sn target thymocytes (7.5×10^5 cells in 0.10 ml) were incubated for 15 min at 37 C guinea pig complement (0.075 ml) was added and the mixture was further incubated for 30 min at 37 C. The diluent was barbiturate buffer μ 0.15 pH 7.4 containing 1.5×10^{-4} M $CaCl_2$, $5 \times 10^{-}$ M $MgCl_2$ and 10 per cent heat inactivated foetal bovine serum. The ^{51}Cr-release into the cell free supernatant was measured in a Biospan TM scintillation detector.

Preparation of $F(ab')_2$ Fragments

Mouse IgG was partly purified by precipitating two volumes of serum with one volume of a saturated $(NH_4)_2SO_4$ solution, pH 7.0. The dialysed IgG solution (15–20 mg/ml) was digested with pepsin (twice crystallized, 1 60 000 Sigma Chemical Co.) for 24 h in 0.2 M acetate buffer pH 4.4 at 37 C. The enzyme to IgG ratio was 1.5 100 (W/W). The enzymatic activity was stopped by adjusting pH to 7.5. The digested material was fractionated on a Sephadex G-100 column (1.4 × 170 cm) equilibrated with 0.5 M NaCl buffered with 0.1 M tris-HCl pH 8.0 plus 2 per cent n-butanol at 4 C. Fractions from the descending part of the major peak, by marker substances and electron microscopy shown to contain $F(ab')_2$ fragments, were collected, concentrated to 4 or 8 mg/ml dialysed against phosphate (0.01 M) buffered physiological saline and stored at −20 C. The cytotoxicity of the $F(ab')_2$ preparations against A/Sn thymocytes was less than 1 per cent of the activity in the original alloantiserum. Pre-incubation of the target cells with the same $F(ab')_2$ preparations reduced the subsequent whole A.CA anti-A/Sn serum induced cytotoxicity by ≥90 per cent.

Purification and ^{125}I-labelling of Mouse IgG

Mouse IgG was isolated from normal ACA and ACA anti A/Sn serum by precipitation twice with neutralized 40 per cent saturated $(NH_4)_2SO_4$ solution at +4 C.

The precipitate was dissolved and dialysed against labelling buffer (0.05 M sodium-phosphate buffer pH 7.0) and after the protein content was determined the IgG was labelled with ^{125}I using the lactoperoxidase method. The labelled protein was separated from free iodide on a small Sephadex G-25 fine column saturated with 0.5 ml 2 per cent albumin in a 0.04 M sodium-phosphate buffer pH 7.4 containing 0.15 M NaCl as elution medium. Fractions of about 0.6 ml were collected, the ^{125}I activity was measured in a Nuclear Chicago Biospan TM scintillation detector and the peak fractions were pooled.

RESULTS

Specific Binding of Alloantibodies in Cardiac Grafts

Ten A.CA mice received allogeneic A/Sn heart/transplants in one ear and syngeneic grafts in the other. On the sixth day after transplantation, 5 mice were given i.m. injection in the right thigh of ^{125}I labelled IgG from normal A.CA serum and 5 mice ^{125}I labelled IgG from A.CA anti-A/Sn serum. The dose per mouse was 70 μg (4.3 $\mu Ci/\mu g$ IgG). All recipients had functioning grafts. Mice in both groups were killed 3, 26 and 73 hours after the IgG injection, the transplants and the recipients' own hearts were excised, cut into small pieces and washed with 0.9 per cent NaCl solution until the ^{125}I activity in the wash fluid was negligible. The tissue was digested with 0.75 per cent trypsin in PBS pH 7.4 for 24 hours at 37 C. The tissue-associated isotope activity in all samples was measured on the same day in a Nuclear-Chicago Biospan TM scintillation detector.

The kinetics involved in the binding of the A.CA anti A/Sn antibodies to the allografts was rather slow (Fig. 1). However the radio-

Specificity of the Enhancement Effect

Administration of normal A.CA serum or A/Sn anti-A.CA serum to A.CA recipients had no effect on A/Sn heart transplant survival (mean 11.4 days, range 9–13 days). In contrast, i.m. injection of 50 µl A.CA anti-A/Sn serum on days 0, 6, 12 etc. exerted a clear enhancement effect (mean survival 21.5 days, range 16–26 days, $t_{(1)}$ = 8.99**) (Fig. 2). A slight, but significant ($t_{(\ )}$ =

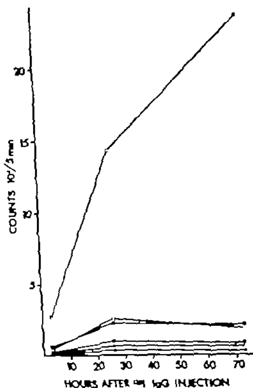


Fig. 1. Specific binding of ¹²⁵I IgG alloantibodies in A.CA recipients. Ten recipients with functioning grafts received an i.m. injection with 70 µg ¹²⁵I IgG on the 6th day after transplantation. IgG-antibody fraction from A.CA anti-A/Sn serum bound to allogeneic A/Sn heart transplants (□), syngeneic heart transplants (○) and to recipients own hearts (△). IgG-fraction from normal A.CA serum bound to allogeneic A/Sn heart transplants (■), syngeneic heart transplants (●) and to recipients own hearts (▲).

activity associated with the allografts was on the average 15 times higher than that associated with syngeneic grafts when determined 73 hours after IgG administration. Resorption of injected ¹²⁵I labelled IgG alloantibodies was tested by measuring and comparing the ¹²⁵I-activity in the thigh muscles on both sides 100 hours after the ¹²⁵I IgG injection. The right thigh, into which the injection was given, contained 0.10 per cent of the activity administered in total, the other thigh muscle containing 0.05 per cent of the activity.

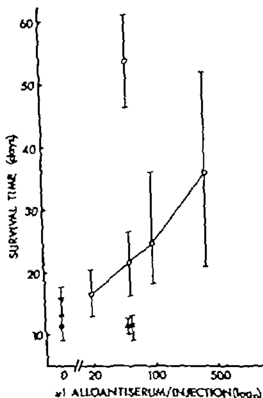


Fig. 2. Mean survival times of heterotopic A/Sn split heart grafts in A.CA recipients receiving different treatments: no immunosuppressive treatment (●); 20 mg prednisone/kg every 3rd day after transplantation (▼); normal A.CA serum i.m. on days 0, 6, 12 etc. post-transplantation (▲); A/Sn anti-A.CA serum i.m. on days 0, 6, 12 etc. post-transplantation (■); A.CA anti-A/Sn serum i.m. on days 4, 10, 16 etc. post-transplantation (○); and A.CA anti-A/Sn serum i.m. on days 4, 10, 16 etc. post-transplantation plus 20 mg prednisone/kg every 3rd day (□). Crossbars represent range of data for each group (8 to 11 mice) from which average was taken.

3 16***) enhancement by A.CA anti A/Sn serum, 50 μl given i.m. each 6th day was observed also in the C3H → A.CA combination. Since the antiserum covered only a part of the major H 2 incompatibilities in this combination this modest effect may be expected.

Synergistic Effect of Alloantibodies or F(ab)₂ and Prednisone on Allograft Survival

A CA recipients receiving i.p injections of prednisone (20 mg/kg) on every 3rd day showed on the average only 4 days prolonged graft survival, while treatment with 20 to 400 μl of the A.CA anti A/Sn serum every 6th day caused a more pronounced dose dependent prolongation of graft survival (Fig 2) A marked potentiation of graft enhancement could be achieved by combining these two treatments (Fig 2) Comparison between treatment with A.CA anti A/Sn serum only (50 μl/injection) and the same serum plus prednisone gave a highly significant t value ($t_{(18)} = 8.00^{***}$) The enhancement effect of F(ab)₂ preparations was potentiated similarly by supporting prednisone injections although the effect was less dramatic (Fig 3)

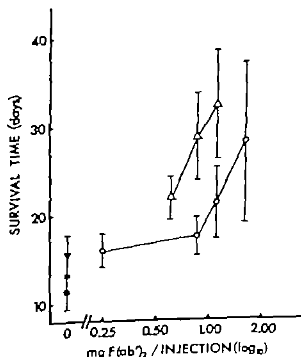


Fig 3 Survival times of heterotopic A/Sn split heart grafts in A.CA recipients receiving from 0.25 to 1.6 mg A.CA anti A/Sn F(ab) i.m. on days 0 6 12 etc. post-transplantation (○) treatment with the same F(ab)₂ preparation plus 20 mg prednisone/kg every 3rd day after transplantation (Δ) prednisone only (▼) and no immunosuppressive treatment (●) Each mean value is based on 10 to 12 determinations and crossbars represent ± 2 SD

No Protection of Graft after in Vitro *Incubation of Heart Tissue with Enhancing Antiserum or F(ab)₂*

Incubation *in vitro* of sectioned and washed hearts with A.CA anti A/Sn serum or its F(ab)₂ fragments for 10 min at 37° C caused slightly reduced graft survival times (9.6 to 10.6 days) The shortest survival times were seen when the complement in the alloantiserum was not heat inactivated (Table 1) *In vitro* incubation with F(ab)₂ followed by deposition of Spongostan, soaked with the F(ab)₂ preparation close to the transplant in the s.c. pouch had no significant effect on transplant survival In contrast, recipients receiving four weekly i.p injections (0.05 ml) of the same alloantiserum or F(ab)₂ preparation showed enhanced graft survival (21 and 18 days, respectively)

Passive Enhancement Effective after Antigen Release but Reduced after Onset of Electrical Activity of Transplant

Treatment with a single dose of alloantiserum was more effective on day 4 post transplantation than on day 0 (Table 2) However a further delay of the treatment to day 6 resulted in a highly heterogeneous graft survival (range 10 to 28 days) and a reduced mean survival In order to be effective, passive enhancement apparently had to be applied prior to days 5 to 6 post transplantation, when electrical activity of transplant begins.

Cytotoxic Antibody Levels in Sera of A CA Recipients

Serum samples were collected from various groups of A.CA 5 recipients one to two weeks

TABLE 1 *Effect of* In Vitro *I cubation of Sectioned Hearts with Alloantiserum or F(ab) Fragments on G aft Survival*

Treatment	No. of transplants	Mean survival and range (days)
None	9	11 4 (9–13)
Heat inactivated A CA anti-A/Sn serum	6	10.5 (7–12)
Not heat inactivated A CA anti-A/Sn serum	6	9 6 (7–11)
A CA anti-A/Sn F(ab) incubation§	8	10.6 (9–11)
A.CA anti-A/Sn F(ab') incubation and spongostan†	10	12.2 (12–14)
A CA anti-A/Sn serum, injection‡	11	21.0 (16–26)
A CA anti-A/Sn F(ab')$_{2}$, injection 0.85 mg/ml∂	12	17.6 (14–20)

Sectioned hearts incubated in vitro for 10 min with alloantiserum diluted 1 10

§ Sectioned hearts incubated in vitro for 10 min in a F(ab) sol tion (4 mg protein/ml)

† Small pieces of spongostan were immersed in F(ab) solution (8 mg/ml) and placed on both sides of the transplants in the pouch.

‡ 0 05 ml of serum given i.m on days 0, 6 12, 18 etc. after transplantation.

∂ Given m. t 6 days interval after transplantation starting on day 0.

after that all animals in the group had rejected their transplants. The F(ab)$_{2}$ preparations, administered to the recipients in some of these groups, possessed less than 1 per cent of the cytotoxic antibody titre in the original serum. In the group of recipients receiving alloantiserum, treatment with enhancing serum was terminated 14 days prior to bleeding. The cytotoxic antibody titres to A/Sn thymocytes of the pooled sera in all the groups receiving immunosuppressive treat ment ranged from 16 to 64. The highest antibody titre (64) was found in sera from mice demonstrating the longest graft survival. The animals not receiving immunosuppression had a mean titre of only 8 but it should be noted that they were bled earlier after transplanta tion.

DISCUSSION

It is evident that the administration of whole alloantiserum, invariably containing enhanc ing as well as cytotoxic antibodies, in certain combinations could be expected to produce damage to the graft (4 6 9 16) Death from alloantiserum-mediated damage of renal allografts has also been observed in certain rat strain combinations (4)

French (6) could induce hyperacute rejec tion of rat renal allografts by adding complement to an alloantiserum which enhanced on its own. Furthermore, *Myburgh & Smit* (15) using baboons, noted in their liver allograft model that treatment with a potent, enhancing alloantiserum occasionally was followed by florid rejections. These rejections were not seen when F(ab) from the same

TABLE 2. *Passive Enhancement Effect of Different Treatment Schedule*

Treatment A.CA anti-A/Sn serum	No. of transplants	Mean survival and range (days)
Gr 1 one inj. (0.1 ml) day 0	10	15.3 (13–19)
G 2, one inj. (0 1 ml) day 4	10	20.2 (19–25)
Gr 3, inj. (0 05 ml) days 6 12, 18 etc.	11	17.3 (10–28)
Gr 4 inj. (0.05 ml) days 0, 6, 12 etc.	11	24 7 (19–30)

Comparison between groups 3 and 4 $t_{(m)} = 3.77$**

alloantiserum was used to induce limited prolonged liver allograft survival

One of the objectives of the present study was to investigate whether passive enhancement could be induced by virtually non-cytotoxic F(ab)₂ when strongly H 2 incompatible cardiac grafts were transplanted. The demonstrated enhancement effect was strongly dependent upon the dose of F(ab)₂ administered. Graft survival increased considerably when a certain dose level was exceeded but the stronger enhancement effect was accompanied by a greater spread of the survival values. With the support of prednisone ≥1 mg F(ab)₂/injection prepared from serum with an antibody titre of 512 gave a highly significant enhancement of murine hearts grafted across this strong H 2 barrier In an attempt to extrapolate from the mouse model to clinical transplantation—assuming 10 times lower antibody titres in the HLA sera—it would appear that >100 mg F(ab)₂/kg should be used

Non-specific immunosuppression with prednisone acted synergistically with enhancing alloantiserum or F(ab)₂. This effect was most pronounced when alloantiserum was used. In particular it was the occasionally occurring early rejections in the 3rd post transplantation week, observed when only F(ab)₂ was used, which could be avoided by the combined treatment. A potentiation of renal allograft enhancement in rats has been observed also after combined treatment with enhancing alloantiserum and ALS (1 4 14) and prolonged graft survival due to the combined effect of enhancing antiserum and x irradiation of recipients was reported by *Jeckel et al* (10)

It has been reported (13 17) that encouraging results might be obtained by pretreatment of the organ with antidonor IgG fragments (F(ab)₂) Other investigators, however have not been able to control hyper acute rejection of human renal grafts by F(ab)₂ pretreatment of the organ (2)

It was tested in the present study whether any efferent protection of the transplant could be obtained by *in vitro* incubation with alloantiserum or F(ab)₂ fragments prior to transplantation No such effect was observed whether heat inactivated alloantiserum or F(ab)₂ were used In contrast, there was a tendency towards reduced transplant survival particularly when non heated alloantiserum was used, compatible with a C-mediated damage to the transplant

In the present system, optimal enhancement effect of a single alloantiserum injection was observed when the injection was given 3–4 days after transplantation, that is, after antigen release but before full vascular reconstitution of the transplant. Thus, afferent enhancement by blocking of alloantigen, did not appear to be an important mechanism The inability to maintain an enhancement effect in spite of continuous administration of rather large doses of alloantibodies and a demonstrated strong specific binding of these alloantibodies in the cardiac graft further speaks against a simple efferent mechanism In cases of skin and kidney transplants, a rapid binding of alloantibodies to antigenic sites in the graft has been described earlier (11 14)

Passive enhancement appears to be most effective in immediately vascularized allografts (23) If the potency of passively administered alloantibodies is to be fully utilized, it seems important that vascular reconstitution precedes lymphatic reconstitution. This, together with the fact that hearts appear to be more vulnerable to an immune attack than kidneys may explain why it is rather difficult to achieve indefinite survival of free foetal heart allografts by passive enhancement.

Observations in this study suggested that recipients with considerably prolonged graft survival—due to treatment with F(ab)₂—had developed slightly higher cytotoxic antibody titres than recipients who rejected earlier It is possible that these gradually increasing cytotoxic antibody titres represented one of the factors which, in the course of time, made it increasingly difficult to maintain enhancement in spite of continued administration of alloantibody F(ab)₂.

Several factors such as mode of grafting functional diversity of the graft, antigen mass, antigen density on the individual cells and possibly also the presence of tissue specific alloantigens have influence on the survival time of a graft.

It would appear however that the importance of tissue-specific alloantigens is limited (22) particularly when their specificities are not determined by genes within the MHC. This notion is supported by the observation that the specific unresponsiveness in recipients of long-term surviving kidney grafts also extends to skin grafts from the same donor strain (18). Any significantly different effects on heart allograft survival were not observed in the present study when the enhancing effect of alloantisera raised against heart and spleen cells, respectively were compared at the same *in vitro* cytotoxicity levels.

A high degree of antibody reactivity against MHC antigens can be removed from an enhancing serum without markedly affecting its enhancing activity (3 19). One interpretation of these results is that it is not necessary in passive enhancement to cover all of the MHC specificities involved in the incompatibility (5). It was suggested also in the present study that a modest but significant enhancement effect can be achieved in a strain combination (C3H → A.CA) where the alloantiserum (A.CA anti-A/Sn) covers only a part of the H 2 incompatibilities.

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REFERENCES

1 *Batchelor J R Fabre J & Morris, P J* Passive enhancement of kidney allografts potentiation with antithymocyte serum. Transplantation 13 610–613 1972

2 *Cerman J L Aashman N Porter K A Andre G I atrals S Putnam C W Porter M Pen I & Starzl T E.* Unsuccessful attempts to control hyperacute rejection of human renal homografts with F(ab) and citrate organ pretreatment. Transplantation 16 60–65 1973

3 *Decker, D A. L. & Alkin B J.* What abrogates heart transpla t rejection in immunological enhancement? N ture 247 294–297 1974

4 *Fabre J W & Morris P J.:* Passive enhancement of homozygous renal allografts in the rat. Transplantation 18 429–435 1974

5. *Fabre J W & Morris, P J:* Passive enhancement of rat renal allografts with only partial cover of the incompatible Ag-B specificities. Transplantation 18 436–442 1974

6. *F en k, U E.* The early effects of alloantibody and complement on rat kidney allografts. Transplantation 13 447–451 1972.

7 *F en k M E & Batchelor J R* Enhancement of renal allografts in rats and man. Transplant. Rev 13 115–141 1972.

8 *Heron I.* Suppression of cardiac allograft rejection in adult rats by pretreatment with bone marrow cells. Immunological enhancement. Acta path. microbiol scand. Sect. B, 80 41 48, 1972.

9 *Holter A R McKearn T J New M R., Fitch F W & Stuart F P.:* Renal transplantation in the rabbit. I Development of a model for study of hyperacute rejection and immunological enhancement. Transplantation 13 244–249 1972.

10. *Jeekel J J McKenzie I F C & Winn H J* Immunological enhancement of skin grafts in the mouse. J Immunol. 108 1017–1024 1972.

11 *Jones J M., Peter H H & Feldman J D* Binding in vi of enhancing antibodies to skin allografts and specific allogeneic tissues. J Immunol. 108 301–309 1972.

12. *Kissmeyer-Nielsen F., Olsen S., Petersen P F & Fjeldborg O.:* Hyperacute rejection of kidney allografts, associated with pre-existing humoral antibodies against donor cells. Lancet ii. 662–665 1966.

13 *Kobayashi K Hricko G., Lakl, P Hunacker L., Patel, R. Reisner G S & Burtch A. G* Hyperacute renal allograft rejection in presensitized monkeys. Surg. Forum 22 246–248, 1971

14 *Morris R J & Lu as Z J* Immunological enhancement of rat kidney grafts Evidence for peripheral action of homologous antiserum. Transpl. Proc. 3 697–700, 1971

15 *Myburg, J A & Smit J A* Passive and active enhancement in baboon liver allografting. Transplantation 14 227–235 1972.

16 *Olsen, J O* The role of hyperimmune alloantiserum in allograft survival. Immunology 25 881–889 1973

17 *Shaipanich T., Vanwijck, R. R., Kon J.-P., Lakl, P., Busch G J & Wilson, R. E.* Enhancement of rat renal allografts with F(ab)

fragment of donor-specific antikidney serum. Surgery *70* 113–121 1971

18. *Skoskiewicz M Chase C Winn H J & Russell P S* Kidney transplants between mice of graded immunogenetic diversity Transpl Proc. *5* 721–725 1973
19. *Staines N A Guy K & Davies D A* Passive enhancement of mouse skin allografts. Specificity of the antiserum for major histocompatibility complex antigens. Transplantation *18* 192–195 1974
20. *Svehag S E & Schilling W* Synergism between alloantibodies or F(ab')$_2$ and prednisone in the survival of murine split heart allografts. Transplantation *15* 345–347 1973
21. *Svehag S E, Harner P Schilling, W & Falksveden L.* Purification and in vitro properties of antihuman and antimacaca thymocyte IgG comparison of prolonged and pulse immunization, and the use of macaca thymocytes to conserve human antigen. Transplantation *11* 274–287 1971
22. *Tilney N L. & Bell P R F.* Studies on enhancement of cardiac and renal allografts in the rat. Transplantation *18* 31 37 1974
23. *Wustrack K O Gruber R P & Lucas Z J* Immunological enhancement of skin allografts in the rat. Transplantation *19* 156–165 1975

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AN EXAMINATION OF THE "STAR-PHENOMENON" A THREE COMPONENT IMMUNOPRECIPITATION INVOLVING STAPHYLOCOCCAL PROTEIN A

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Grov A & Endresen, C. An examination of the "star-phenomenon" three component immunoprecipitation involving staphylococcal protein A. Acta path. microbiol scand. Sect. C, 84 333-336, 1976

The co-precipitation called star-phenomenon occurred between the three component system protein A, an IgG forming soluble complexes with protein A and F(ab')$_2$-preparations of human IgG guinea pig IgG or rabbit anti-staphylococcal IgG Co-precipitation also occurred if the IgG was replaced by normal human Fc fragments. On a protein A column the human F(ab') -preparation was separated into a major non-reactive and a minor reactive fraction. Only the latter contained Fc-structures, these being isolated on an anti-Fc column and found to belong to undigested IgG The "star"-forming, protein A reactive F(ab') -fragments were washed through the anti-Fc column. The F(ab') fraction from rabbit anti-staphylococcal IgG contained no Fc-structures and only a small portion containing anti-protein A activity was active in co-precipitation.

Key words Staphylococcal protein A "star-phenomenon"

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The "star-phenomenon" the unique co-precipitation described by *Kronvall & Williams* (8) occurs between the three component system Staphylococcal protein A, anti-protein A F(ab') fragments, and normal rabbit IgG Both the bivalency of immune F(ab') and the Fc mediated protein A reactivity were found to be essential in this context.

Lind & Mansa (10) and *Lind* (9) have later shown that star formation follows a set-up of protein A, an IgG forming soluble complexes with protein A (Fc reactivity) and F(ab') fragments from any serum that precipitates protein A. They explained the reactivity of the F(ab') fragments as being due to antibody specificity directed against hidden determinants of IgG revealed by protein A's interaction with the Fc-region. They also claimed that the use of F(ab') of anti protein A or anti-staphylococcal sera, did not involve antibodies to protein A itself contradicting the assumption of *Kronvall & Williams* (8)

The finding that the F(ab') fragments could be replaced by human IgM and IgA that interact with protein A through Fc (3, 4) prompted us to re-examine the mechanism behind the "star"-formation.

MATERIALS AND METHODS

Protein A Protein A was prepared from *Staphylococcus aureus* strain Cowan I as described in (5)

Sera. Normal human serum was a pool of sera from 20 healthy blood donors, and both normal rabbit and normal guinea pig sera were also pools of 15–20 individual sera Rabbit antisera to *S aureus* strain Cowan I were produced by intravenous injection of formalin-killed bacteria (11) and rabbit antisera to human $F(ab')_2$, Fc and pFc and to guinea pig Fc as described in (1) A goat anti-rabbit Fc serum was obtained by absorption of a goat *anti rabbit IgG (Hyland, Belgium) by* $F(ab')_2$ fragments of normal rabbit IgG

Immunoglobulin preparations IgG was prepared by precipitation in 1.33 M $(NH_4)_2SO_4$ followed by fractionation on DEAE-cellulose (12) Protein concentrations were measured as before (3)

$F(ab')_2$ fragments IgG was digested with pepsin (Sigma USA) in an 0 1 M acetate buffer pH 4 0 containing 5 mM NaCl for 18 h at 37 C, the substrate to enzyme ratio being 100.3 The digest *was then fractionated on a column of Sephadex* G-150 equilibrated with 0 1 M Tris-HCl pH 7.8 containing 0 2 M NaCl and 2 mM EDTA Na

Fab fragments were prepared by reduction of isolated $F(ab')_2$ fragments in 0 2 M 2 mercaptoethanol at pH 8.0 and room temperature in the dark for 18 h alkylation in 0.3 M iodoacetamide *for 3 h at 37 C, and finally dialysis against saline* buffered to pH 8 0

Fc fragments were isolated from papain digests of IgG on a column of DEAE-cellulose (1?) gel filtrated on Sephadex G-150 as described above and tested for purity against specific antisera

Immunological tests Double diffusion in agar inhibition of precipitation and tests for a direct co-precipitation and the star formation were carried out as previously (3) Indirect haemagglu*tination using tanned sheep erythrocytes, was per*formed as described in (6)

Immunosorbents Columns of protein A coupled to sepharose 4B (Pharmacia, Sweden) were prepared and used as previously (?) An immunosorbent of IgG from an antiserum to human Fc fragments was prepared and used similarly

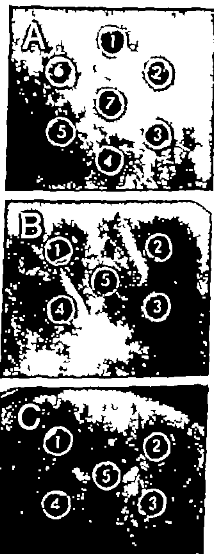


Fig 1 Co-precipitations in agar A shows star formation between protein A (7) normal rabbit IgG (1) and (4) and either human $F(ab')_2$ (5) or $F(ab')$ from rabbit anti-staphylococcal IgG (6) The protein A non-reactive fractions of the two latter preparations, in (3) and (2) respectively B shows direct co-precipitation between protein A (5) and 1 1 mixtures of normal rabbit serum and $F(ab')$ from rabbit anti-staphylococcal IgG (8 mg ml) (?) and the human $F(ab')_2$ preparation (10 mg ml) (4) The protein A non-reactive fraction of the same mixed with normal rabbit serum in (1) and (3) respectively C shows "star formation between protein A (5) and (4) $F(ab')_2$ from rabbit anti-staphylococcal IgG (5 and normal human Fc fragments at 5.2 mg/ml (1) and ? 6 mg ml (2)

RESULTS

The star' precipitation starts at a central point the "arms appearing later on $F(ab')$ fragments from normal human IgG produced a "star together with protein A and normal rabbit IgG (Fig 1A) A star was also produced when the human $F(ab')_2$ fragments were replaced by concentrated $F(ab')$ fragments of normal guinea pig IgG or $F(ab')$ from rabbit anti-staphylococcal IgG (Fig 1 A) but not when $F(ab')_2$ fragments from normal rabbit IgG were used "Star forma

tion was also observed when normal rabbit IgG was replaced by normal human Fc fragments (Fig. 1 C) In this case the "star" was somewhat different in appearance, usually having only two arms

To find out if there were any fragments in the human F(ab) preparation interacting directly with protein A, the preparation was applied to a protein A-Sepharose column. Most of the material (about 95 per cent) was washed out with phosphate buffered saline, but about 5 per cent was retarded and eluted with 3 M NaSCN The protein A reacting fraction gave co-precipitation (Fig 1 B) and star -formation whereas the non-reacting fraction neither gave a star" nor co-precipitation in concentrations up to 20 mg per ml. In double diffusion the protein A reacting material precipitated with both anti-human Fc and anti-human pFc' sera, whereas the protein A non-reacting fraction showed no precipitation line against any of these sera. Further pepsin digestion of the protein A reacting fraction gave approximately 50-50 of reacting and non-reacting material each time. These results indicate that the active fraction in the human F(ab)$_2$-preparation participating in star"-formation contains insufficiently digested IgG molecules which are rather resistant to pepsin.

However when the protein A reacting material was applied to an immunosorbent column of IgG from a rabbit antiserum to human Fc fragments, the star"-forming fraction was washed through. This fraction contained no Fc-determinants, but was fixed to sepharose-linked protein A which had never been in contact with any immunoglobulins. After reduction and alkylation the fraction (Fab) lost its ability to co-precipitate, but was still fixed to protein A as demonstrated both by the immunosorbent column and by inhibition of precipitation between protein A and normal human serum. This shows that the star" forming F(ab) fragments have antibody activity against protein A The fraction bound to the anti-Fc column was found to react with both anti-Fab and anti Fc sera and to be undigested IgG Upon concentration this material gave a direct precipitate with protein A.

The F(ab) preparation from IgG of rabbit anti-staphylococcal serum gave "star" formation together with protein A and normal rabbit IgG (or human Fc fragments) (Fig. 1 A and C) The reaction was dependent on a relatively high concentration of the F(ab) preparation, optimal concentration being in the range of 8–10 mg/ml. The material contained no Fc-structures as judged from the lack of interaction with goat anti rabbit Fc serum both in double diffusion and indirect haemagglutination. On a protein A sepharose column the F(ab) material was separated into two portions, a small protein A reactive and a major non-reactive fraction.

The former gave co-precipitation and "star" formation with an optimal concentration in the range of 1–2 mg/ml, the latter fraction was non-reactive to protein A in all tests at concentrations up to 20 mg/ml. The anti-staphylococcal serum thus contained specific antibodies to protein A. The incapability of the protein A non-reacting F(ab) fraction to co-precipitate was not changed after mixing with human Fc fragments. At concentrations of about 5 mg/ml the protein A reacting F(ab) fraction gave a precipitation line with protein A in agar

DISCUSSION

There seems to be no doubt about the necessity of Fc-mediated soluble complexes in the star"-formation. In contrast to others (8, 10) we found that IgG of normal rabbit serum or an inhibiting myeloma serum could be replaced by Fc fragments of human IgG In this case the lower concentration limit was about 2.6 mg per ml and the shape of the co-precipitation ("star") was more like a v

The crucial point, however seems to be the F(ab) fragments. All experiments showed that the human "F(ab) fraction participating in "star"-formation was fixed to a column of protein A. Since this fraction, in contrast to the non-reacting, contained Fc structures, a Fc-mediated interaction seemed

likely However using an anti Fc column the Fc structures were found to belong to undigested IgG molecules, the "star" forming entity being $F(ab')_2$ fragments directed against protein A. Similarly a small part of the F(ab) fraction isolated from rabbit anti-staphylococcal IgG was shown to originate from specific protein A antibodies. The major parts of all the $F(ab)_2$ preparations were non reactive both on the protein A column and in co-precipitation tests. Neither of the present experiments gave any indication for the presence of antibodies interacting with the IgG of soluble protein A IgG complexes (9)

A specific protein A activity of normal human (and guinea pig) IgG is in accordance with the finding of *Kronvall et al* (7) that carbamylation of a normal precipitating IgG selectively destroyed the γ-globulin structure essential for precipitation without affecting the capacity to combine with protein A. Similarly star' formation with human IgM (3) and IgA (4) which interact with protein A may be due to the presence of specific protein A antibodies of these classes. Reduction and alkylation destroyed the capacity of IgM and IgA to co-precipitate and to produce a "star" whereas the combination with protein A through Fc was maintained The lack of a direct co-precipitation, however is difficult to explain unless Fc mediated soluble complexes of protein A and IgM/IgA are sterically less favourable for interaction with specific anti protein A antibodies of these classes than are soluble protein A IgG complexes. Proper concentrations of the reactants are also highly important in this context.

A direct precipitation between protein A and anti protein A apparentl, depends on a relatively high concentration of the antibodies. Thus, co-precipitations most probably is the usual mechanism not only in normal sera but also in anti-staphylococcal sera having low concentrations of anti protein A antibodies.

REFERENCES

1 *Endresen C* Unpublished work
2. *Grov A* Studies on the interaction between staphylococcal protein A and the Fc-region of Immunoglobulin G Acta path microbiol scand. Sect. A, Suppl. *236* 77–83 1973.
3 *Grov A* Human IgM interacting with staphylococcal protein A. Acta path. microbiol. scand. Sect C, *83* 173–176 1975.
4 *Grov A* Human colostral IgA interacting with staphylococcal protein A. Acta path. microbiol. scand. Sect. C, *84* 71–72, 1976.
5 *Grov A Myklestad B & Oeding P* Immunochemical studies on antigen preparations from *Staphylococcus aureus* 1 Isolation and chemical characterization of antigen A. Acta path. microbiol. scand. *61* 588–596 1964.
6 *Grov A & Oeding, P.*. Serological cross-reactions of tanned erythrocytes sensitized with staphylococcal antigens. Acta path. microbiol. scand. Sect. B, *79* 539–544 1971
7 *Kronvall G Messner R P & Williams R. C., Jr.*. Immunochemical studies on the interaction between staphylococcal protein A and γG globulin. J Immunol. *105* 1353–1359, 1970
8 *Kronvall G & Williams R C., Jr.*. The star phenomenon, a three component immunoprecipitation involving protein A. Immunochemistry *8* 577–580 1971
9 *Lind I* The formation of antibodies against hidden determinants of autologous IgG during immunization of rabbits with *Staphylococcus aureus* Scand J Immunol. *3* 689–696 1974
10 *Lind I & Mansa B* Immunochemical study of the interaction between staphylococcal protein A, rabbit antistaphylococcal sera, and selected sera from non-immunized animals. Scand J Immunol *3* 147–156 1974
11 *Oeding P* Agglutinability of pyogenic staphylococc at various conditions Acta path. microbiol. scand. *41* 310–324 1957
12. *Rosa G & Nisonoff A* Crystallization of fragment Fab of human IgG myeloma proteins. Biochem Biophys. Res. Commun. *31:* 914–918, 1968.

STUDIES OF THE ANTIGENIC STRUCTURE OF *TRICHOPHYTON RUBRUM* *TRICHOPHYTON MENTAGROPHYTES* *MICROSPORON CANIS* AND *EPIDERMOPHYTON FLOCCOSUM* BY CROSSED IMMUNO-ELECTROPHORESIS

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Christiansen, Aa. H. & Svejgaard, E. Studies of the antigenic structure of *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporon canis* and *Epidermophyton floccosum* by crossed immuno-electrophoresis. Acta path. microbiol. scand. Sect. C, 84: 337–341, 1976.

By means of crossed immuno-electrophoresis, 35 antigens of *T. rubrum*, 26 of *T. mentagrophytes*, 35 of *M. canis*, and 23 of *E. floccosum* has been demonstrated. Tandem crossed immuno-electrophoresis has revealed two antigens common to *T. rubrum* and the other species, which, however, differ within the species. No common antigens has been demonstrated between *T. mentagrophytes*, *M. canis* and *E. floccosum*. The technique for preparation of extracts and production of immune serum is described, and the procedure for the electrophoresis is outlined.

Key words: Dermatophytes; antigenic structure; crossed immuno-electrophoresis.

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Previous studies of various species of pathogenic dermatophytes have mainly been focused upon polysaccharides (4). A study comprising disc electrophoresis of proteins from dermatophytes has revealed 9 compounds from *Microsporon canis* and 14 compounds from *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Epidermophyton floccosum* (8).

Immuno-electrophoresis has been used by *Andrieu et al.* (1) for the analysis of 17 species of dermatophytes. They defined about ten different antigens, several of which showed cross-reactions.

Ito (5, 6) isolated 22 fractions from *T. mentagrophytes var. asteroides* by phenol-water extractions and chromatography.

The present paper describes a gentle method for extraction of antigens, production of antibodies in rabbits, and studies of the patterns of antigens prepared from four pathogenic species of dermatophytes applying crossed immuno-electrophoresis. A similar

method was found to be successful in the production of antigens from *Candida albicans* (2)

By these methods more than 25 different antigens from the four species have been demonstrated indicating an immunochemical structure even more complex than realized before

MATERIAL AND METHODS

Since type cultures of dermatophytes do not exist (10) the following strains isolated in the mycological laboratory of this department were used *T rubrum T mentagrophytes var granulare M canis* and *E floccosum* A spore suspension was made from pure cultures grown on Sabouraud dextrose agar by adding small (1–5 mm) sterile glass balls and sterile water After shaking of the dishes to loosen the spores, the suspension was transferred to bottles containing Sabouraud dextrose broth 2 per cent and incubated at 26 C for 4 weeks. The wet cultures passed a rough homogenization step (Ultra Turrax apparatus) were lyophilized and stored at −20 C until use.

Preparation of Antigens

Three grams of lyophilized material were suspended in 10 ml of distilled water and transferred to a homogenization bottle containing 50 grams of glass beads (dia. 0 45–0.50 mm) and 10 ml distilled water The extraction was performed in a Braun cell homogenizer (MSK) at ?800 rev/min for 6 subsequent periods of 30 seconds using CO_2 as a coolant. The extract was cleared of debris by centrifugation at 1000 *G* for 20 minutes. Thereafter the supernatant was centrifuged at 20000 *G* in a Sorvall centrifuge at 4 C The protein content of the supernatant after this second centrifugation was determined by the method of *Lowry et al* (7) and a second lyophilization was performed. From the dried material and sterile water a solution containing 10 mg protein per ml was made The solution was passed through a sterile filter (Millipore ®GSWP 04700 25 ea GS 0 22 μ) stored in small tubes (1–2 ml) at 80 C and used as antigen Microscopy of the material was performed after each step The cell walls were not broken until after treatment in the MSK

Immunization of Rabbits

For immunization, one ml of the antigen extract was emulgated in one ml Freund's incomplete adjuvant (equal parts v/v) and 0 2 ml (1 mg protein) injected intracutaneously on the shaved back of each animal Injections were given once a week for five weeks, and after this, twice a month for about a year Antibody formation was observed by passive diffusion in agarose gel from the fifth week on. Ten ml blood from an ear vein was used for this purpose and drawn one week after each injection. The animals were divided into four groups of five each receiving the same batch of antigen. Serum of the animals in each group was pooled after omission of poor antibody formers.

Concentration of Serum

To 10 ml serum ?.5 g of $(NH_4)_2SO_4$ was added, and the mixture left at room temperature for 20-24 hours followed by centrifugation. The supernatant containing albumin, transferrin, alpha proteins, and free haemoglobin was discarded. The precipitate was washed twice with 1 75 M $(NH_4)_2SO_4$ and dissolved in 2 ml water By this procedure a 4–5 fold increase of the immunoglobulin concentration was obtained. The serum was frozen and stored in 1–2 ml samples.

Immunological Methods

The antibody formation was controlled by passive diffusion in 1 per cent agarose gel in barbital buffer according to the micromethod of *Wadsworth* (9)

The antigen pattern of the extracts were examined by crossed immuno-electrophoresis in 1 per cent agarose gel (Litex, Glostrup, Denmark) in barbital buffer (pH 8.6 ionic strength 0 02) The first run for separation of antigens was carried out at 10 C applying 10 V/cm for one hour The

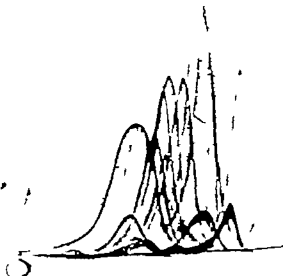


Fig 1 Crossed immuno-electrophoresis using extract from *T rubrum* and homologous immune serum. Thirty-five antigens are shown Site of application of extract Entire lower site to the left. Anode To the right First run, second run at top.



Fig 2 Crossed immuno-electrophoresis with extract from *T mentagrophytes* and homologous immune serum. Twenty-six antigens are shown.



Fig 3 Crossed immuno-electrophoresis with extract from *M canis* and homologous immune serum. Thirty-five antigens are shown.

second run for precipitation in agarose containing immune serum was performed at 10 C applying 2 V/cm for 20 hours. 10 µl antigen solution (100 µg protein) and 0.5 ml antiserum were used for one glass plate, 10 by 10 cm, the thickness of the gel being one mm. When the electrophoresis was completed, the plates were removed from the electrophoresis unit, covered with filter paper and squeezed slightly for 5 minutes. The unprecipitated material was removed by washing with 0.1 M saline for one hour and the salts by washing with distilled water for one hour. Finally the plates were dried and the precipitates stained with Coomassie brilliant blue R in ethanol—glacial acetic acid—water (45 10 45). Excess dye was removed by two washings in dyefree solvent.

Comparative analyses of the antigen patterns of different species was done, using the tandem crossed immuno-electrophoresis, where two antigen solutions from different species are applied side to side in the first run. The antigen solution corresponding to the immune serum was placed closest to the cathode. The interpretation of the result of such an experiment is based upon the possible existence of double peaks which indicate similar antigens from different species.

For details regarding equipment and technique the reader is referred to *Axelsen et* 1973 (3).

RESULTS

The yield of water-soluble antigens in extracts from 3 grams of lyophilized dermatophytes was about 500 mg, including 100 mg of protein.

The antigen pattern of *T rubrum* is shown in Fig 1. Each peak represents one antigen and 35 antigens were recognized. Fig 2 shows the pattern of *T mentagrophytes* 26 antigens were demonstrated. In Fig 3 the pattern of *M canis* shows 35 antigens, and finally Fig 4 shows the pattern of *E. floccosum* with 25 antigens.

Fig. 5 shows the result of a tandem immuno-electrophoresis to reveal antigenic similarities between *T rubrum* and *T mentagrophytes*. Extracts from these fungi were tested against antibody to *T rubrum*. Two double



Fig 4 Crossed immuno-electrophoresis with extract from *E. floccosum* and homologous immune serum. Twenty-five antigens are shown.

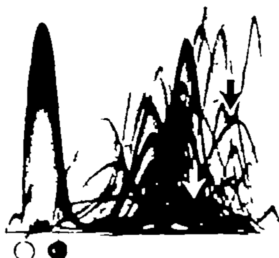


Fig 5 Tandem crossed immuno-electrophoresis comparing extracts from *T rubrum* and *T mentagrophytes* using antibody to *T rubrum.* Two double peaks indicated by arrows show common antigens.

peaks in the anodic area were recorded, thus indicating two similar antigens. This experiment was repeated using antibody to *T mentagrophytes* and again two common antigens were found in the anodic area

When extracts from *M canis* and *E floccosum* were compared with extract from *T rubrum* and antibody to *T rubrum* these species were found to have two antigens in common although these antigens were located in different places on the plate depending on the species in question and suggesting that the similarity of antigens from *T rubrum* and the other species vary from species to species.

So far we have failed to demonstrate antigens common to *T mentagrophytes M canis* and *E floccosum*

DISCUSSION

Using a gentle method for antigen preparation and crossed immuno-electrophoresis a structure of the four species of dermatophytes more complex than realized before was revealed

The disintegration in the ball mill used in this study comprising six periods of 30 seconds was found to be sufficient since further extractions failed to increase the yield of protein in the extracts. However since only about 500 mg per 3 grams of fungi (dry weight) or 17 per cent were rendered soluble by the extraction procedure it is possible that an even greater number of antigens may be present in the undissolved residue Therefore it must be emphasized that e.g the 35 antigens demonstrated in the extract of *T rubrum* are considered to be a minimum antigen yield in this species. The disintegration to occur during preparation of the antigen causes a liberation of proteolytic enzymes, resulting in digestion of the proteins in the extracts. Thus, it was necessary to store the extracts at –80 C to avoid a decrease in the antigen content. Repeated freezings and thawings resulted in a similar decrease in the number of antigens. Hence, the extracts should be stored in small samples of e.g 0.5 ml and used immediately after thawing

It appears from the figures that some precipitates seem to be strong while others seem to be faint This is due either to differences in the immunogenicity of the antigens or to quantitative conditions indicating that a relatively high content of a potent antigen gives a strong precipitate. The faint precipitates are not always demonstrable in serum samples from different animals. It is, therefore preferable to work with pooled immune sera from selected animals with good antibody response Even under standardized conditions, the patterns may vary from one experiment to another but the most distinct peaks are fairly constant in shape and location and by their appearance, enable the investigator to differentiate between different species

In this study two antigens have been demonstrated to be common to *T rubrum* and the other three species of dermatophytes. However the electrophoretic mobility of these antigens differs from one species to another and thus, it seems likely that the common antigens also do so

The fact that the majority of the antigens

are different in the various species may provide additional knowledge of taxonomic characteristics of fungi.

The authors are indebted to Dr N Axelsen Protein Laboratory University of Copenhagen, for valuable advice, and to Mrs. *H Andersen* and Mrs. *S Kahn* for technical assistance.

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REFERENCES

1 *Andrieu S Biguet J & Laloux B* Analyse immunoélectrophorétique comparée des structures antigéniques de 17 especes de dermatophytes. Mycopathologia 34 161–185 1968.

2 *Axelsen N* Antigen—antibody crossed electrophoresis (Laurell) applied to the study of the antigenic structure of *Candida albicans*. Infection and Immunity 5 5.5–527 1971

3. *Axelsen N Krøll, J & Weeke B.* (Eds.): A manual of quantitative immunoelectrophoresis. Scand. J Immunol. Suppl. 1 1973

4 *Grappel, S F., Bishop C T & Blank F* Immunology of dermatophytes and dermatophytosis. Bact. Rev 38 222 250, 1974

5 *Ito Y* On the immunologically active substances of the dermatophytes. I J Invest. Derm. 45 275–284 1965

6. *Ito Y..* On the immunologically active substances of the dermatophytes. II Ibid. 45 285–294 1965

7 *Lowry O H., Rosenbrough, N N., Farr A L. & Randall, R J.* Protein measurement with the folin phenol reagent. J biol. Chem. 193 265–275 1951

8. *Schechter Y., Landau, J W., Dabrowa, N & Newcomer V D* Comparative disc electrophoretic studies of proteins from dermatophytes. Sabouraudia 5 144–149 1966

9 *Wadsworth C.* A slide microtechnique for the analysis of immune precipitates in gel. Int. Arch. Allergy 10 355-360, 1957

10 *de Vries G A.*, Centraalbureau oor Schimmelcultures, Holland. Personal communication.

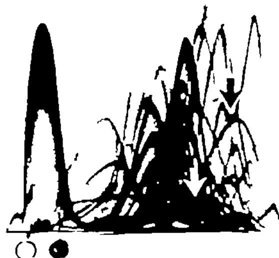


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TABLE 1 *Survival Time and Urine Volume from Two Control and One T ated Experimental Groups*

	Survival of graft time min mean (limits)	Urine volume ml mean (limits)
Group 1 Control 13 experiments	20 (10–90)	5 (0–32)
Group 2 Serum control 7 experiments	32 (10–75)	4 (1 10)
Group 3 Treatment 5 experiments	>170 (60–>300)	>160 (5–>400)

In group 3 only the observed figures are shown although 3 of the animals certainly could have show excellent kidney appearance and urine production if the experiments had been prolonged. In two of the animals, bleeding and haemorrhagic shock terminated the experiment while the kidney was not rejected

of Freunds complete adjuvans. The immunization procedure was followed by crossed immune electrophoresis

The anti cat serum was pplied 1) v to the recipient cat in 4–8 ml aliquots one hour before establishment of recirculation 2) in the *perfusion* fluid used for flushing the donor kidney 3–5 ml anti cat serum per 100 ml of perfusion fluid.

Three experimental groups were investigated

Group 1 Controls. In 13 *animals*, renal xenotransplantation was performed using rabbit as donor and cat as recipient. The *appearance of the* transplanted kidney and the urine volume were observed and the morphology studied by histology When the kidney became cyanotic it was judged as rejected.

Group 2. Serum control. In 7 animals the same procedure as in group 1 was performed, the only difference being that normal duck serum was administered in the perfusion fluid and to the recipient.

Group 3 Treatment with anti cat serum. In 5 animals the same procedure as in group 2 was performed, except that the serum contained anti cat serum antibodies.

A striking prolongation of survival of the graft was observed in group 3 treated with anti donor serum antibodies, as seen from Table 1 Survival for several hours of the graft with brisk diuresis was observed in contrast to the control groups here survival lasted for few minutes and urine formation was almost absent.

Histology revealed marked difference between the treated group (group 3) and the other groups A detailed report concerning the histology *will* be published later

Xenograft rejection and hyperacute rejection ex hibit number of similar traits as far as time sequences, histology antibody and complement depletion etc. are concerned. Nevertheless it has not been proved that the two processes are idea tical *until the minute details, for example*, in nature of involved antibodies. In this brief report, however we *will deal with* the two *events as if* they are of the same origin.

I the prevention *of* hyperacute or *xenograft* rejection, short lasting success has been achieved by removal of antibodies (*either preformed or* raised by immunization) or of complement from the blood of the recipient (5 1 4 3 2) *This* removal of antibodies has been achieved by differ ent means but to our knowledge the method described here—injection of antibodies against preformed, normally existing serum antibodies—has never been proposed or employed, probably because it may be difficult to obtain the correct dose of antiserum, a dose requiring protection of the graft, at the same time avoiding toxic response of the recipient. The latter effect may be responsible for the outcome of some of the present experiments, termed "haemorrhagic shock" Other possible causes for prolongation of graft survival are interference by anti-idiotypic antibodies and formation of immune complexes capable of complement binding

The possibilities *involved* in the method tempt us to follow the trace. Fractionation of the potent serum may filter unwanted side *effects* away and facilitate usage of the fractions containing the wanted effect. Such experiments are now in preparation. Usage of the methods in the clinical routine is obviously far away

I conclusion w has prolonged survival of renal xenografts from rabbit to cat for a *consider*

Acta path. microbiol scand Sect. C, *84* 342–344 1976

BRIEF REPORT

PROLONGATION OF XENOGRAFT SURVIVAL BY INFUSION OF HETEROLOGOUS ANTIBODIES AGAINST RECIPIENT SERUM

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Kemp E., Kemp G., Svendsen P Nielsen, E., Buhl, M R. Jacobsen, I A. & Lundborg C. J Prolongation of xenograft survival by infusion of heterologous antibodies against recipient serum Acta path microbiol. scand. Sect. C *84* 342–344 1976

Survival of renal xenografts from rabbit to cat has been prolonged by several hours by injection of antibodies against cat serum into the recipient In control experiments the transplanted rabbit kidney was rejected by the cat in a few minutes.

Key words Xenotransplantation renal transplantation antiserum treatment.

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Rejection of a xenotransplant is probably caused by preformed recipient serum antibodies which interact with donor cell antigens. Thus, a cell damaging process is starting and especially the cells of the endothelium of the small vessels become injured, leading to thrombosis in some minutes.

This sequence of events might be inhibited by infusion into the recip ent of antibodies raised against the serum of the recip ent and thus against the preformed antibodies of the recipient This hypothesis was tested n a renal rabbit to cat xenotransplantation model and found to hold true

25 renal xenotransplantations were performed. Kidneys from rabbits were transplanted to cats The donor rabbit was pre treated with chlorpromazine heparin and furosemide before nephrectomy

The left kidney from the donor rabbit was flushed with a slightly modified Collins sol tion immediately after removal. The kidney was then stored in a refrigerator at 4 C until implanted

Cats of either sex weighing 2–4 kg were used as recipients.

Bilateral nephrectomy was performed through an abdominal transperitoneal midline incision from the xiphoid process to the symphysis.

The aorta and inferior vena cava were dissected between two lumbar arteries and veins, respectively distal to the original renal vessels.

The inferior vena cava was clamped above and below between two lumbar veins with one pair of curved forceps. A five mm incision was made on the front side of the vein and the graft vein was anastomosed end to side with continuous 8-0 nylon. The aorta was then clamped in the same manner as the vein and opened by removing an elipsoid patch (2 × 1 mm) immediately distal to the venous anastomosis End to side anastomosis of the graft artery was subsequently performed with continuous 8-0 nylon

The ureter was left free in the abdominal cavity One hour before establishment of recirculation, azathioprine 25 mg and metylprednisolon, 100 mg were injected intravenously

Antiserum again t cat serum antibodies was raised by ntraperitoneal injections to ducks on day 0 21 and 42 of 1 ml cat serum diluted with saline to a protein content of 3.5 mg/ml + 0 1 ml

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IMMUNOLOGICAL MEMORY AFTER PRIMING WITH A THYMUS INDEPENDENT ANTIGEN NIP FICOLL

4-hydroxy-5-iodo-3-nitrophenylacetyl Coupled to Polymer of Sucrose and Epichlorhydrin

M. HURME

Department of Serology and Bacteriology, University of Helsinki, Helsinki, Finland

Hurme, M. Immunological memory after priming with a thymus independent antigen, NIP Ficoll. 4-hydroxy-5-iodo-3-nitrophenylacetyl coupled to polymer of sucrose and epichlorhydrin. Acta path. microbiol. scand. Sect. C, 84: 345-352, 1976.

The capacity of mouse spleen fragments to mount an anti-NIP (4-hydroxy-5-iodo-3-nitrophenylacetyl) response in vitro was studied. The fragments came from unprimed, NIP Ficoll (polymer of sucrose and epichlorhydrin) or NIP-CG (chicken globulin) primed mice. Unprimed spleen fragments from C57BL/6 mice gave a good anti-NIP response to NIP Ficoll, whereas CBA fragments did not. Priming with NIP Ficoll made CBA fragments responsive and enhanced slightly the response of C57BL/6 fragments when stimulated with the same antigen. This memory effect could be seen only after a small priming dose. Priming the mice with NIP Ficoll made their spleen fragments responsive to a protein conjugate of NIP (NIP-CG) but this effect was seen only after priming with a high dose. The antibody class distribution and the kinetics of the appearance of different immunoglobulin classes were similar in the primary and secondary responses *in vitro*. The peak responses of IgM, IgA and IgG were reached on day eight and the relative amount of IgG was the same in the primary and in the secondary responses. Spleen fragments derived from NIP-CG primed mice produced more IgG anti-NIP antibodies than fragments derived from untreated mice when immunized *in vitro* with NIP-Ficoll. The amount of IgG was, however, much higher when these fragments were challenged with the homologous antigen, NIP-CG.

Key words: Immunological memory; thymus independent antigen; NIP Ficoll; priming.

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Antibody response to most antigens requires collaboration between T and B cells, but certain antigens, e.g. pneumococcal polysaccharide type III, polymerized bacterial flagellin, polyvinylpyrrolidone, levan (polyfructose), Ficoll (polymer of sucrose and epichlorhydrin) can induce an antibody response in the absence of T cells (1, 10, 13, 22, 23). Haptenated derivatives of these thymus independent antigens induce an anti-hapten antibody production also without T cell help. The B cell populations stimulated in this situation by the hapten and the carrier are

able time by injection of antibodies against cat serum into the recipient

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References 1 *Belitsky P Popovtzer M Corman J Launois B & Porter K A* Transplantation *15* 248, 1973 —2 *Kemp E, Kemp G Jacobsen I A & Lundborg C J* Unpublished work—3 *Land W Hammer C., Fiedler L & Brendel, W.,* Res. exp. Med. *158* 1 1972.—4 *Shons A R & Najarian J S* Trans. Amer Soc. Artif. Int. Organs *20* 562, 1974 —5 *Simpson K M Bunch, D L, Amemiya H Boehmig H J Wilson, C B Dixon F J Coburg A J., Hathaway W E, Giles G R & Starzl T E.,* Surgery *68* 77 1970.

Acta path. microbiol. scand. Sect. C, 84 345–352, 1976

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known to be separate and non interacting (9, 19) The actual mechanism of B cell activation by these antigens is unknown and several models have been proposed (for ref see 30)

The antibody class elicited by an immunization with a thymus independent antigen is usually IgM but sometimes small amounts of other classes have been detected, like IgA and IgG in anti pneumococcal polysaccharide response (4) and IgG in anti-DNP Ficoll response (23, 29) The antibody class distribution of spleen fragment culture to NIP pneumococcal polysaccharide is exceptional, because at the peak of the response the anti NIP antibodies are totally of IgA class (24)

Immunization with a thymus independent antigen has been reported to fail in stimulating significant production of memory cells (3 6 12 13 14 19 20 29) Even in those cases where memory has been found the priming effect has been small (29) or has manifested itself only as a capacity to respond to smaller, non immunogenic doses (12)

In this study the following features of Ficoll coupled to hapten NIP are characterized (i) the primary response *in vivo* and *in vitro* in spleen fragment culture (ii) the capacity to induce and stimulate memory cells and (iii) the antibody class in these responses.

MATERIALS AND METHODS

Antigens. The antigens used were the NIP (4-hydroxy 5-iodo-3-nitrophenylacetyl) derivative of Ficoll (Pharmacia Uppsala, Sweden) (kindly provided by Dr *O Mäkelä*) The coupling was done by the cyanogen bromide activation method (?) Briefly to 50 mg Ficoll in 3 ml H_2O was added 0.05 N NaOH until pH was 10 0 This solution was poured into 40 mg CNBr and pH was kept at 10 0–10.5 for 30 min with 0 5 N NaOH Then 0.5 ml 6 × 10 ² M NIP-ethylendiamine was added The mixture was incubated for 4 h at room temperature and then overnight at 4 C. The conjugate was dialysed first against 0 05 M ethanol amine, then several times against 5 per cent $NaHCO_3$ The conjugate had 26 moles of NIP/mole of Ficoll (average molecular weight 400 000) NIP coupled to chicken globulin (CG prepared from fresh chicken serum by $(NH_4)_2SO_4$ precipitation) was also used. The coupling was made by the method of *Brownstone et al.* (7) This conjugate had 10 moles of NIP/mole of CG.

Animals and immunizations. Female C57BL/6 or CBA mice, 2 to 4 months old, were used. Some mice were immunized with different, intraperitoneally applied doses of NIP Ficoll in saline (vol. 0.5 ml) or with intraperitoneally applied doses of 100 µg alum precipitated NIP-CG (plus 10⁹ *Bordetella pertussis* bacteria) NIP Ficoll immunized mice were bled from the tail vein 7 14 and 28 days after immunization and then used for in vitro cultures.

Culture technique The Millipore filter well technique for organ culture of mouse spleen fragments was used as described earlier (27) with slight modifications (24) The spleen fragments were prepared with McIlwaine tissue chopper machine and their size was about 1 mm³ The culture medium, Eagle's minimum essential medium plus 10 per cent agamma horse serum (North American Biologicals Inc., batch no. 102 889) as changed every two days. Thus, the antibody titre of day 8 designates the antibody content of medium used for culture on days 6 to 8. Antigens were placed directly on each fragment in a volume of 0.01 ml. Media from 5 fragments were pooled for titration Different antigen doses were tested with fragments derived from the same spleen.

Assay of antibodies Anti-NIP antibodies in culture fluids or sera were measured by NIP-T⁴ phage inactivation method (21) The sera were also titrated in the presence of 2-mercaptoethanol. The titres in sucrose gradient fractions were measured by the same method but using a more sensitive NIP-caproic acid coupled bacteriophage (5)

Sucrose gradient ultracentrifugation. 0.1–0.3 ml samples of culture media or sera in a suitable dilution were centrifuged through a continuous and linear 5–20 per cent sucrose gradient in 12 ml tubes in rotor SW 41 Ti of a Beckman L3 50 ultracentrifuge. The running time was 14 h (39,000 rev/min) A total of 25 fractions was collected and titrated. Using this centrifugation protocol, IgM is found in tubes 3–6 (from the bottom) and IgG in tubes 16–18. IgA is located between IgM and IgG (16 18) Before centrifugation some samples were pretreated by adding 2 5 µl of rabbit anti-mouse IgA to 150–200 µl of culture medium. The mixture was incubated for 1 h at 37 C and stored at 4 C until centrifugation This treatment completely inhibits the appearance of IgA peaks (16, 18, 24) and it had no suppressive effect on anti-NIP activity in 19S or 7S peaks. Anti IgA antiserum (kindly provided by Dr *O Mäkelä*) was prepared recently in this laboratory by immunizing rabbits with α-chain purified from MOPC 315 myeloma ascites fluid (24) The antiserum was rendered monospecific for IgA by passage through

TABLE 1 *The Primary Anti-NIP (4-hydroxy-5-iodo-3-nitrophenylacetyl) Response to NIP-Ficoll* In vivo

Mouse strain	Dose of NIP Ficoll	Anti-NIP titre Day 7	Day 14	Day 28
C57BL/6	100 µg	6.10 (.06)	5.98 (.13)	5.63 (.12)
		4.29 (.11)§	4.59 (.06)	4.23 (.22)
	1 µg	5.85 (.04)	5.53 (.04)	5.42 (.03)
		3.70 (.12)	3.90 (.11)	3.77 (.15)
	0.1 µg	3.63 (.19)	3.84 (.20)	3.49 (.16)
		<3.00	<3.00	<3.00
CBA	100 µg	4.90 (.07)	5.15 (.08)	4.62 (.06)
		<3.00	<3.00	<3.00
	1 µg	4.91 (.03)	4.90 (.05)	4.38 (.06)
		<3.00	<3.00	<3.00
	0.1 µg	3.48 (.05)	3.36 (.08)	3.35 (.04)
		<3.00	<3.00	<3.00

total titre, log mean (standard error) of 3 mice.
§ 2-mercaptoethanol resistant titre.

mouse IgG Sepharose 4B immunosorbent column. The undiluted antiserum was immunoelectrophoretically monospecific for IgA when tested against normal mouse serum and against the MOPC 315 ascites fluid.

RESULTS

The Primary Response to NIP-Ficoll in Vivo

CBA and C57BL/6 mice were immunized intraperitoneally with different doses of NIP

TABLE 2 *The Anti-NIP (4-hydroxy-5-iodo-3-nitrophenylacetyl) Response of Spleen Fragments Derived from NIP-Ficoll Primed or Unprimed C57BL/6 or CBA Mice*

Priming dose	Antigen in vitro	Anti-NIP titre on day 8 C57BL/6	CBA
—	20 µg NIP Ficoll	1.96 (.19)	<1.00
	2 µg	3.23 (.27)	<1.00
	0.2 µg	3.13 (.28)	<1.00
	0.02 µg	2.16 (.16)	<1.00
	—	<1.00	<1.00
100 µg	2 µg NIP Ficoll	2.29 (.18)	<1.00
	0.2 µg	2.19 (.24)	<1.00
	0.02 µg	2.26 (.23)	<1.00
	0.2 µg NIP-CG§	2.29 (.25)	2.08 (.23)
1 µg	2 µg NIP Ficoll	not done	1.98 (.16)
	0.2 µg		1.99 (.08)
	0.02 µg		1.68 (.05)
	0.2 µg NIP-CG		1.85 (.12)
0.1 µg	2 µg NIP Ficoll	3.28 (.33)	1.95 (.31)
	0.2 µg	3.63 (.19)	2.11 (.33)
	0.02 µg	2.20 (.29)	1.73 (.20)
	0.2 µg NIP-CG	<1.00	<1.00

log mean (standard error) of 4–8 experiments.
§ NIP coupled to chicken globulin.

Ficoll. At day 7 the response was already almost maximal in both strains but C57BL/6 mice produced titres more than 10 times higher (Table 1) As regards the tested doses 100 μg and 1 μg induced a good anti NIP production but 0 1 μg dose did not induce titres significantly above the natural anti NIP titres of these strains. The amounts of 2 mercaptoethanol resistant antibodies (IgG) were low and in the sucrose gradient centrifugations almost all of the anti NIP activity was in the 19S area.

The Anti NIP Response of Spleen Fragments Derived from NIP Ficoll Primed or Unprimed Mice

Different amounts of NIP Ficoll were added to spleen fragments derived from unprimed C57BL/6 mice and anti NIP production was maximal in culture fluid collected on day 8 The highest titres were obtained with 0 2 or 2 μg doses of NIP Ficoll (Table 2) Spleen fragments from CBA mice did not give a response above that of control cultures (no added antigen)

C57BL/6 and CBA mice were primed with 100 μg, 1 μg and 0 1 μg doses of NIP Ficoll and their spleens were taken 1–2 months later for fragment culture. Fragments were stimulated with different doses of NIP Ficoll or NIP-CG Table 2 shows that NIP Ficoll could now induce a response in CBA mouse spleen fragments, but only if the priming dose had been small (less than 100 μg) Increased responses in C57BL/6 mouse spleen fragments could only be detected after a small priming dose and even then the memory effect was small The priming changed slightly the antigen dose-response curve of C57BL/6 spleens (0 2 μg dose now induced a better response than 2 μg dose)

NIP-CG which does not induce a primary anti NIP response in the spleen fragment culture (16, 24) could now elicit a significant anti NIP response but this was true only if a high priming dose of NIP Ficoll was used.

The anti NIP antibodies produced in the primary and secondary responses were studied by sucrose gradient centrifugation NIP Ficoll stimulated fragments derived from unprimed C57BL/6 mice produced at the be-

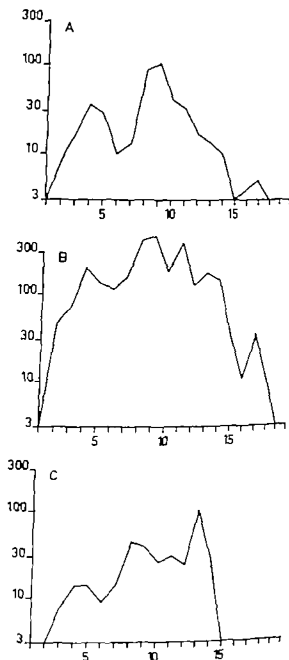


Fig 1 Sucrose gradient centrifugation of spleen fragment culture media. Fragments derived from C57BL/6 mice were immunized *in vitro* with 0.2 μg NIP Ficoll (4-hydroxy 5-iodo-3-nitrophenylacetyl coupled to Ficoll) Day 6 (A) day 8 (B) and day 10 (C) samples. The anti-NIP titres in this experiment were 330 on day 6, 10300 on day 8 and 2600 on day 10 Abscissa fraction number from the bottom, ordinate anti-NIP titre

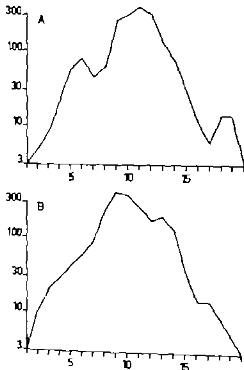


Fig 2 Sucrose gradient centrifugation of spleen fragment culture media. Fragments derived from CBA mice primed 1 month previously with 1 μg NIP Ficoll (4-hydroxy-5-iodo-3-nitrophenylacetyl coupled to Ficoll) were challenged in vitro with 2 μg NIP Ficoll (A) or with 0.2 μg NIP-CG (chicken globulin) (B). Day 8 samples. Abscissa fraction number from the bottom, ordinate anti-NIP titre.

ginning of the responses (day 6 culture media) IgM, IgA and IgG antibodies, but IgA was in a great majority. At the peak of the responses (day 8) the class distribution was similar and later on only the smallest peak (IgG) had fallen under detection level. Representative centrifugation patterns are shown in Fig. 1. In some centrifugations, the samples were pretreated with anti-IgA antiserum which abolished the anti-NIP activity between the 19S area (tubes 3–6) and the 7S area (tubes 16–18).

The antibody class distribution in the memory responses was the same as in the primary response whether the antigen was homologous or heterologous (NIP Ficoll or NIP-CG) (Fig 2)

TABLE 3 *The Anti-NIP (4-hydroxy-5-iodo-3-nitrophenylacetyl) Response of Spleen Fragments Derived from C57BL/6 and CBA Mice Primed 1–2 Months Previously with NIP-CG (NIP Coupled to Chicken Globulin)*

Antigen in vitro	Anti-NIP titre on day 8 C57BL/6	CBA
20 μg NIP-Ficoll	1.81 (.20)	1.65 (.17)
2 μg	2.74 (.21)	2.43 (.46)
0.2 μg	3.23 (.20)	3.06 (.30)
0.02 μg	2.44 (.11)	2.93 (.22)
0.2 μg NIP-CG	4.38 (.13)	4.10 (.24)
–	<1.00	<1.00

log mean (standard error) of 4–8 experiments.

The Secondary Response of Spleen Fragments Derived from NIP-CG Primed Mice

C57BL/6 and CBA mice were primed with 100 μg of NIP-CG and their spleen were taken 1–2 months later for fragment culture. When spleen fragments were stimulated with the optimum dose of NIP-CG a high secondary response was noticed (Table 3). If the challenging antigen was NIP Ficoll, a good anti-NIP production was obtained in both mouse strains, but it was not higher than the primary response to NIP Ficoll in C57BL/6 spleen fragments.

The class of anti NIP produced after NIP-CG challenge was mainly IgG but a minor component of IgA could also be detected (Fig 3). After NIP Ficoll challenge the IgG peak was much smaller but it was still higher than that in the primary response to NIP Ficoll. The antibody class distribution was the same in both mouse strains.

DISCUSSION

These data show that priming with a thymus independent antigen, NIP Ficoll, induces a weak but distinct immunological memory. Spleen fragments from untreated CBA mice did not respond to this antigen, but frag

Ficoll. At day 7 the response was already almost maximal in both strains but C57BL/6 mice produced titres more than 10 times higher (Table 1) As regards the tested doses, 100 μg and 1 μg induced a good anti NIP production but 0 1 μg dose did not induce titres significantly above the natural anti NIP titres of these strains. The amounts of 2 mercaptoethanol resistant antibodies (IgG) were low and in the sucrose gradient centrifugations almost all of the anti NIP activity was in the 19S area.

The Anti NIP Response of Spleen Fragments Derived from NIP Ficoll Primed or Unprimed Mice

Different amounts of NIP Ficoll were added to spleen fragments derived from unprimed C57BL/6 mice and anti NIP production was maximal in culture fluid collected on day 8. The highest titres were obtained with 0 2 or 2 μg doses of NIP Ficoll (Table 2) Spleen fragments from CBA mice did not give a response above that of control cultures (no added antigen)

C57BL/6 and CBA mice were primed with 100 μg, 1 μg and 0 1 μg doses of NIP Ficoll and their spleens were taken 1–2 months later for fragment culture. Fragments were stimulated with different doses of NIP Ficoll or NIP-CG Table 2 shows that NIP Ficoll could now induce a response in CBA mouse spleen fragments, but only if the priming dose had been small (less than 100 μg) Increased responses in C57BL/6 mouse spleen fragments could only be detected after a small priming dose and even then the memory effect was small. The priming changed slightly the antigen dose-response curve of C57BL/6 spleens (0.2 μg dose now induced a better response than 2 μg dose)

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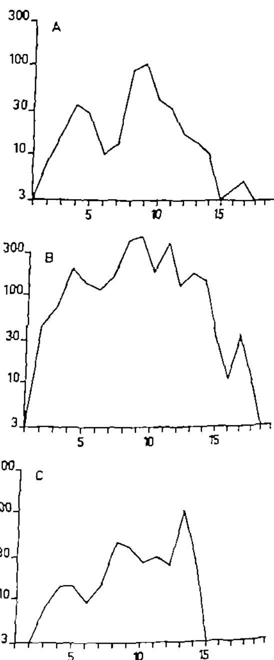


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The anti-NIP antibodies produced in the primary and secondary responses were studied by sucrose gradient centrifugation NIP Ficoll stimulated fragments derived from unprimed C57BL/6 mice produced at the be-

Ficoll conjugate which has been reported to induce a high IgG production (29) 7S antibody production seems to be more easily paralyzed than 19S antibody production by thymus independent antigens, as detected by subsequent challenge with a thymus dependent conjugate (11) If thymus independent antigens are used, the weakness of memory is mainly due to the absence of IgG B memory cells and thus, the reported slight increases in IgM titres are compatible with the weak IgM B cell memory reported in other experimental conditions (28) In these experiments, there was not either evidence of increased IgG responses.

The antibody classes produced by unprimed or NIP Ficoll primed spleen fragments after NIP Ficoll immunization were IgM, IgA and IgG The high IgA production in this culture system has been a constant finding in primary responses to thymus independent antigens, like NIP coupled to pneumococcal polysaccharide (24) In responses to hapten-protein conjugates in fragments derived from carrier primed mice (16) or in true secondary responses (15) The kinetics of the appearance of different immunoglobulin classes after immunization with a thymus independent antigen is different from the responses to thymus dependent antigens. In these experiments, the three main immunoglobulin classes were all detected already in the beginning of the response and peak responses in each class were obtained simultaneously This is in agreement with the kinetics reported to be involved in anti-pneumococcal polysaccharide responses (4) or in anti-hapten responses after immunization with haptenated Ficoll (29) The same kind of kinetics was observed also when NIP-CG primed spleen fragments were stimulated with NIP Ficoll (data not stated) In this case, the relative amount of IgG was higher than in the primary response to NIP Ficoll which shows that this thymus independent antigen is also capable of stimulating some IgG memory cells, but to a much lower extent than the homologous conjugate (NIP-CG) Thus, T cell help makes it possible to stimulate more IgG B memory cells and in this situation the IgG response is also lasting longer (15)

The memory cell populations induced by NIP-CG were dissimilar in C57BL/6 and CBA mouse strains. The challenge with NIP-CG *in vitro* showed that C57BL/6 memory cells responded to a much narrower dose range than CBA memory cells (data not stated) This finding may be in agreement with the earlier findings obtained in this laboratory (17) findings which showed that C57BL/6 mice have a strong affinity clone for anti-NIP production. Thus, memory cells derived from this clone would be rather homogenous and could be stimulated with the same antigen dose. The absence of this clone in CBA mice could explain the unresponsiveness of their spleen fragments *in vitro* and also the lower anti-NIP titres *in vivo*. The experiments in which this question is to be studied are now in progress.

The author wishes to thank Dr *O Mäkelä* in whose laboratory this work was done, for his advice and critical revision of the manuscript. Mrs. *Kaija Rytsy* gav skilful technical assistance.

REFERENCES

1 *Andersson B & Blomgren H.*, Evidence for thymus-independent humoral antibody production in mice against polyvinyl pyrrolidone and *E. coli* lipopolysaccharide. Cell. Immunol. 2 411–424 1971

2. *Axen R. & Ernback, S.*, Chemical fixation of enzymes to cyanogen halide activ ted polysaccharide carrier Eur J Biochem. *18* 351–360 1971

3 *Baker B. J., Stashak, P W Amsbaugh, D F & Prescott B.* Characterization of the antibody response to type III pneumococcal polysaccharide at the cellular level I Dose-response studies nd the effect of prior immunization on the magnitude of the antibody response. Immunology 20 469–480 1971

4 *Barthold D R., Prescott B., Stashak P W., Amsbaugh, D F & Baker P J.*: Regulation of the antibody response to type III pneumococcal polysaccharide III. Role of regulatory T cells in the development of an IgG and IgA antibody response J Immunol. *112* 1042–1050 1974.

5. *Becker M & Mäkelä, O.*, Modification of

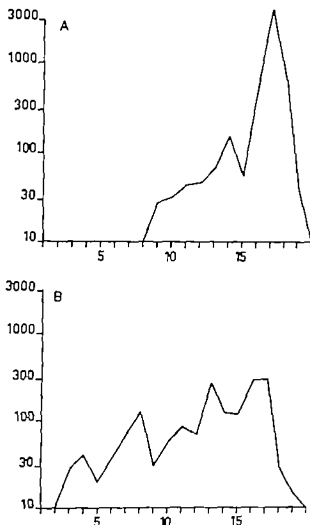


Fig 3 Sucrose gradient centrifugation of spleen fragment culture media. Fragments derived from CBA mice primed 1–2 months previously with 100 μg NIP-CG (4 hydroxy 5-iodo-3 nitrophenyl acetyl coupled to chicken globulin) were immunized *in vitro* with 0 2 μg NIP-CG (A) or 0.2 μg NIP-Ficoll (B) Day 8 samples. Abscissa fraction number from the bottom, ordinate anti NIP titre.

ments of NIP Ficoll primed mice did so Spleen fragments of C57BL/6 mice gave a good primary response to this antigen and spleens of primed mice responded only slightly better In both mouse strains, a small priming dose was better for memory induction than a large dose. Further evidence of the existence of memory cells was the anti NIP response elicited by NIP-CG in fragments derived from NIP Ficoll primed but not from unimmunized mice. This effect was seen only after a high dose of NIP Ficoll in priming

In the earlier reports, where memory after immunization with a thymus independent antigen has not been found, several explanations have been presented. It has been suggested that in the absence of T cell help, thymus independent antigens may drive B cells in the terminal differentiation without establishment of memory B cells (14) Thus, memory formation is possible if thymus independent antigens are introduced in a thymus dependent form like pneumococcal polysaccharide type III coupled to sheep red blood cells (6) or as a part of whole bacteria (25) However it seems that generation of B memory cells requires only minimal T cell activity because thymus dependent antigens are capable of inducing B memory cells in thymus-lacking (nude) mice (8, 26) The lack of the secondary response is not due to pre-existing antibody (14 20)

In these experiments, only a small priming dose of NIP Ficoll (1 μg or 0 1 μg) could induce memory cells which were responsive to the same antigen *in vitro* The reason for the lack of memory after high dose priming is not the pre-existing anti NIP antibodies because the *in vivo* titres after 100 μg and 1 μg doses were equally high and because no response was obtained after 100 μg priming dose even with a high dose of *in vitro* antigen The reason could rather be the retained antigen which is able to inhibit the secondary stimulation. The long persistence is a feature common of thymus independent antigens. Memory cells which could be stimulated by a thymus dependent NIP-conjugate, were induced only by a high NIP Ficoll dose in priming The explanation of this may be that high dose priming tolerized cells with high affinity receptors and accordingly the induced memory cells with low affinity receptors could be stimulated only by the T cell mediated mechanisms

In those cases where memory has been found after priming with a thymus independent antigen the increase has been mainly due to IgM antibodies, even with haptenated

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ANTIBODIES TO SYNTHETIC ACTH IN ASTHMATIC AND RHEUMATIC PATIENTS

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Jonsson, J. & Pegelow, K.-O.: Antibodies to synthetic ACTH in asthmatic and rheumatic patients. Acta path. microbiol. scand. Sect. C, 84: 353–364, 1976.

The prevalence of antibodies against synthetic ACTH preparations and porcine gamma globulin was investigated in 29 asthmatic patients, in 28 rheumatic patients, and in 63 normal subjects of both sexes. Agglutinating antibodies were examined by a passive haemagglutination test and IgE antibodies by radio-immuno assay. The incidence of agglutinating reactions against Synacthén® and porcine gamma globulin was significantly higher in the asthmatic and rheumatic groups than in the group of normal subjects. Titres were generally low, attaining supposedly pathological levels (≥ 1/200) in about 10 per cent of the asthmatic patients and in 20–40 per cent of the rheumatic patients. In asthmatic patients, the incidence of ACTH specific IgE was higher than that in the normal group. This difference was not statistically significant in patients on routine treatment but it became statistically significant after ACTH booster treatment. In rheumatic patients the incidence of IgE antibodies against ACTH was significantly lower than that in the normal group. The incidence of agglutinating antibodies against vasopressin in asthmatic and rheumatic patients was also increased as compared with that in normal subjects and the titres were positively correlated to those against ACTH.

Key words: Synthetic ACTH; antibodies; asthmatic, rheumatic patients.

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Allergic reactions against ACTH have been described almost since the introduction of this hormone into therapy. During the first decade of its use most of the reactions were ascribed to a sensitization against contaminating animal protein in the natural pituitary extract (1, 3, 18). Later when more purified extracts came into use, reactions against species characters, expressed by the amino acids 25–33 (10, 14, 19), of the natural hormone molecule, were distinguished. Therefore synthetic ACTH preparations composed of the first 24 amino acids of the natural molecule, tetracosactid, representing the hormone specific part of the molecule were taken into therapeutic use and expected not to entail any risk of sensitization. However allergic responses have occasionally occurred even to such preparations. Several cases of allergic shock induced by tetracosactid have been reported and antibodies against this substance have been demonstrated in asthma patients as well as in cases of connective tissue disease treated with ACTH (3, 6, 7, 9, 10, 12, 13, 15, 16, 21, 25). It therefore seemed to be of interest to estimate the incidence of antibodies to tetracosactid, octacosactid and nat

bacteriophage with hapten ε aminocaproyl N hydroxysuccinimide esters increased sensitivity for immunoassay Immunochemistry *12* 329–331 1975

5. *Braley-Mullen H* Regulatory role of T cells in IgG antibody formation and immune memory to type III pneumococcal polysaccharide J Immunol. *113* 1909–1920 1974

7 *Brownstone A Mitchison N A & Pitt Rivers R* Chemical and serological studies with an iodine-containing synthetic immunological determinant 4-hydroxy 3 iodo-5-nitrophenylacetic acid (NIP) and related compounds. Immunology *10* 465–480 1966.

Diamantenstein T & Blitstein Willinger E. T-cell independent development of B memory cells. Eur J Immunol *4* 830–832 1974

Feldmann M Induction of immunity and tolerance in vitro by hapten-protein conjugates II Carrier independence of the response to dinitrophenylated polymerized flagellin Eur J Immunol. *2* 130–137 1972

Feldmann M & Basten A The relationship between antigenic structure and the requirement for thymus-derived cells in the immune response. J exp Med *134* 103–119 1971

Del Guercio P Poirier M F & Thobie N 7S class-restricted hapten-specific paralysis by injection of thymus-independent hapten-carrier conjugate. J Immunol. *115* 1239–124? 1975

Del Guercio P Thobie N & Poirier M F IgM anamnestic immune response to the haptenic determinant DNP on a thymus-independent carrier J Immunol *112* 427–430 1974

Howard J G Cellular events in the induction and loss of tolerance to pneumococcal polysaccharides. Transplant. Rev *8* 50–75 1972

Howard J G & Courtenay B M Induction of B cell tolerance to polysaccharides by exhaustive immunization and during immunosuppression with cyclophosphamide Eur J Immunol *4* 603–608 1974

Hurme M & Kontiainen S IgA in secondary antihapten responses in vitro Scand J Immunol In press.

Hurme M Nakamura I Kaartinen M & Mäkelä O Antibody response by cultured spleen fragments from carrier primed mice to hapten-protein conjugates. Scand J Immunol *4* 229–234 1975

Imanishi T & Mäkelä O Inheritance of antibody specificity I Anti (4 hydroxy-3-nitrophenyl)acetyl of the mouse primary response J exp. Med. *140* 1498–1510 1974

18. *Kaartinen M Hurme M & Mäkelä O* Evidence for oligomeric IgA production by peripheral rat lymph nodes. Nature (Lond.) *252* 329–330 1974

19 *Klaus G G B & Humphrey J H* The immunological properties of haptens coupled to thymus-independent carrier molecules I. Characteristics of the immune response to dinitrophenyl lysine-substituted pneumococcal polysaccharide (SIII) and levan. Eur J Immunol. *4* 370–377 1974

20 *Lerman S P Romano T J Mond, J J Heidelberger M & Thorbecke G J* Induction of primary and inhibition of secondary antibody response to hapten by hapten conjugates of type III pneumococcal polysaccharide Cell. Immunol. *15* 321–335 1975.

21 *Mäkelä O* Assay of anti hapten antibody with the aid of hapten-coupled bacteriophage. Immunology *10* 81–86 1966

22 *Miranda J J* Studies on immunological paralysis IV The immunogenicity and tolerogenicity of levan (polyfructose) in mice. Immunology *23* 829–842, 1972

23 *Mosier D E., Johnson B. M Paul, W E & McMaster P R B* Cellular requirements for the primary in vitro antibody response to DNP Ficoll. J exp. Med. *139* 1334–1360, 1974

24 *Nakamura I Ray A & Mäkelä, O* Oligomeric IgA the major component of the in vitro primary response of mouse spleen fragments. J exp Med. *138* 973–988, 1973.

25 *Paul W E. Benacerraf B. Siskind G W Goidl E A & Reisfeld R A* The anamnestic antibody response to type III specific pneumococcal polysaccharide J exp. Med. *130* 77–89 1969

26 *Roelants G E & Askonas B. A* Immunological B memory in thymus deprived mice Nature (New Biol) (Lond.) *239* 63–64 1972

27 *Segal S Globerson A Feldman M Haimovich J & Sela M* In vitro induction of a primary response to the dinitrophenyl determinant. J exp Med. *131* 93–99 1970.

28 *Seppälä I Hurme M Sarvas H & Mäkelä O* The role of B cell memory in secondary IgG and IgM responses. Scand. J Immunol In press

29 *Sharon R McMaster P R B Kask A M Owens J D & Paul W E* DNP-lys-Ficoll A T independent antigen which elicits both IgM and IgG anti-DNP antibody-secreting cells. J Immunol *114* 1585–1589 1975.

30 *Möller G* (Ed) Concepts of B lymphocyte activation Transplant Rev *23* 1–265 1975

Materials

^{125}I-tetracosactid (Radiochemical Centre Amersham, Buckinghamshire, England) was obtained from ACTH-immuno assay kits (Wellcome Research Laboratories, Beckenham, Kent, England). The preparations were stated to have a specific radioactivity of about 200 μCi/μg. Their antigen concentration determined by haemagglutination-inhibition tests (5) was adjusted to 10 ng/ml.

Sephadex anti-IgE complex was taken from kits of the commercial Phadebas IgE test (Pharmacia Ltd Uppsala, Sweden) in use for routine RIST test. It was used in the same amount and concentration as that recommended by the manufacturer for the RIST test i.e. usually 1–2 mg of gel in 1 ml.

Procedur

0 1 ml of the ^{125}I-ACTH solution was mixed with 0 1 ml of undiluted test serum.

Control tubes containing negativ control serum + ^{125}I labelled ACTH were included

After one hour t room temperature and two hours at +4 C, 1 0 ml Sephadex-anti-IgE complex was added and the tubes containing the mixture ere incubated for 2 hours at room temperature in rack rotating slowly around ts longer horizontal axis.

The Sephadex particles were centrifuged for 5 minutes at 1000 × g and washed three times with 2 ml saline. After the last centrifugation, the pellet as resuspended in a drop of saline and its radioactivity was measured in Nuclear Chicago counter

In the main experimental series, the background non-specific adsorption of labelled ACTH to the Sephadex particles was not disturbing I some experiments performed 18 months later it was found to ary with different batches of anti-IgE Sephadex, some batches giving considerable background absorption Pre-incubation of the gel with low molecular protein, preferably thyrotropin (Actyron® see Results) reduced the non-specific adsorption of ACTH in such cases

Calculations

The amount of labelled ACTH attached to the anti-IgE-Sephadex complex was determined from standard curv plotting log counts per minute (cpm) against log pg of ACTH. This relationship was approximately linear The calculation was pursued on the basis of the following assumptions

Mol w Tetracosactid	2933.49
IgE	196000 00

Combining proportions IgE = 2 ACTH.

The formula used for calculation was therefore the following:

$$\text{pg IgG} = \frac{\text{pg Tetracosactid}}{2 \text{ MoLw Tetracosactid}} \text{ MoLw IgE.}$$

The pg IgE figure in the formula divided by 100 was called one unit (U) One unit thus came t correspond to about 1 ng. The applied formula could therefore be simplified to

Units IgE/ml = ng IgE/ml = 0.3341 pg Tetracosactid.

RAST Test for the Estimation of IgE Anti-ACTH (23)

ACTH was coupled to cyanogen bromide activ ted Sepharose as described by the manufacturer (Pharmacia Ltd Uppsala, Sweden) 0 5 ml of the ACTH coupled gel containing 1 mg of gel/ml was incubated with 0 5 ml of the unknown sample for 18 hours at room temperature under ertical rotation of the test tubes. After separation of the test serum and three washings with 2 ml 0 15 M saline with 1 per cent Tween 20, the gel had 0 1 ml ^{125}I labelled anti-IgE (Pharmacia Ltd Uppsala, Sweden) with a scintillation activity of about 40000 cpm dded to it and incubation and washings were repeated as described abov

The radioactivity of the gel was then measured. Counts twice that of the negative control were taken to indicate a specific reaction.

Chromato-elect phoresis

This test was performed as described by *Landon et al.* (12, 13) with the exception that serum was used instead of plasma. 0 2 ml of serum diluted 1 2 in 0 025 M phosphate buffer pH 7.5 containing human serum albumin 5 mg/ml and mercaptoethanol 0 5 per cent and 120 pg ^{125}I-tetracosactid in 0 2 ml of the same buffer were incubated for 4 days at +4 C. 0 1 ml was then removed immediately for the electrophoresis The remainder was first acidified to pH 2.0 by 1 N HCl for 10 minutes.

The electrophoresis (13) was performed on Whatman N 3 MC filter paper 37 × 35 mm using a barbital buffer pH 8 6 μ = 0 1 The runs were performed for 10 hours at 220 V and 3 mA/strip of filter paper

The protein reactions were identified by staining with naphtalene black. The strips were divided into 0.5 cm pieces the radiation activity of which was measured in a Nuclear Chicago γ counter

Absorption of Sera

Absorption was performed by incubating one olume of undiluted serum with one olume of packed BDB treated erythrocytes coated with the antigen for 1 hour at 37 C and 1 hour t 20 C.

ural ACTH in patients with atopic conditions and with collagen disease. The findings from such a study will be reported below

MATERIAL AND METHODS

Clinic Material

Asthma patients Sera from 29 patients with asthma bronchiale aged between 20 and 70 years were examined. Nine patients randomly picked up from the office routine had been treated for one year or more with Acton prolongatum Twenty patients had been more closely studied (16) before and during one year's treatment with either *Acton®* 25 i.e. 7–8 times during 3 weeks followed by Acton prolongatum® 7–8 times during three weeks and finally 100 IE at least once monthly or *Synacthén®* 0 25 mg and Synacthén® Depot 1 mg instead of Acton and Acton prolongatum® respectively but otherwise according to the same schedule

Before this treatment, all the 20 *patients* had been treated for one year or more with Acton prolongatum

Rheumatic patients Sera collected at random from ?8 patients with polyarthritis or systemic connective tissue disease were examined The patients were between ?4 and 76 years of age (mean *age 50 years) The serum samples were usually* drawn during therapy with Acton prolongatum 30 IE/week or more. In some cases refractoriness or hypersensitivity to the preparation had been noted.

Normal subjects Supposedly normal subjects were collected from a group of apparently healthy persons subjected to a careful medical health control examination. Data from this study will be reported elsewhere (16) The sera from, in all 63 persons were chosen to represent both sexes and ages between 20 and 60 years

ANTIGENS AND METHODS

Passive Haemagglutination Tests

Tanned *sheep erythrocytes sensitized* according to *Boyden's* description (4) were used with Acton® and the different immunoglobulins Synacthén® Homactid® and Postacton® were attached to sheep erythrocytes by the bisdiazo benzidin technique described by *Stavitsky & Arquilla* (20)

The following antigens were used *in the recorded* concentrations

Synacthén® (24 tetracosactid 0.25 mg = 25 IE/dry ampoule CIBA-GEIGY AG Basel)	5 µg/ml
Homactid® (octacosactid 0.5 mg = 50 IE/dry ampoule Ferring AB, Malmö)	5 µg/ml
Acton® (30 IE/ml Ferring AB, Malmö)	1.2 I U/ml
Postacton® (Vasopressin 20 IE/ml, Ferring AB, Malmö)	5 µg/ml
Porcine γ-globulin (purchased from The Royal Veterinary College Stockholm)	100 µg/ml
Bovine γ-globulin (Nutritional Biochemical Corporation Cleveland, Ohio)	100 µg/ml
Ovine γ-globulin (domestically prepared from sheep serum by ammonium *sulphate precipitation)*	*100 µg/ml*

The agglutination titrations were performed in 10 × 70 mm serological test tubes and read by the bottom patterns of the sedimented erythrocytes.

Haemagglutination-inhibition tests were performed as described previously (5)

Twofold dilution series of sera were used, beginning at *1/10*

A level of distinction between normal and pathological reactions was chosen (Fig 1) in order that

1 the difference between the reactivity rate in the pathological populations and that in the normal population at this level was near its maximum,
2 the incidence of reactions in the normal population did not exceed 5 per cent at this level.

Mixed Haemadsorption Tests

The procedure followed was a two dimensional *radial agar diffusion* technique previously described (10) Solutions of the antigens mentioned in the paragraph on passive haemagglutination were spread over glass slides, dried and fixed with acetone Agar and test sera were applied by the aid of a plastic template After 40 hours diffusion the latter and the agar layer were removed and antibody attached to the antigen was traced by indicator cells, i anti-globulin coated erythrocytes. The procedure used for the determination of the antibody titres in this system has been described previously (11) Further details may be found in a separate report (16)

Radio-immuno Assay f IgE Anti ACTH

A radio-immuno assay originally employed by *Ishizaka et al.* (8) was tentatively used for this purpose

Principle Anti ACTH of the IgE class present in patients sera was allowed to combine with radio-labelled tetracosactid added in excess. The resulting complexes were collected by an excess of anti-human IgE attached to Sephadex particles. The latter were then separated out by sedimentation and their radioactivity was recorded as a measure of the serum anti-ACTH contents.

Materials

^{125}I-tetracosactid (Radiochemical Centre Amersham, Buckinghamshire, England) was obtained from ACTH-immuno assay kits (Wellcome Research Laboratories, Beckenham, Kent, England). The preparations were stated to have specific radioactivity of about 200 µCi/µg. Their antigen concentration determined by haemagglutination-inhibition tests (5) was adjusted to 10 ng/ml.

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Procedure

0.1 ml of the ^{125}I ACTH solution was mixed with 0.1 ml of undiluted test serum.

Control tubes containing negative control serum + ^{125}I labelled ACTH were included.

After one hour at room temperature and two hours at +4 C, 1.0 ml Sephadex-anti-IgE complex was added and the tubes containing the mixture were incubated for 2 hours at room temperature in rack rotating slowly around its longer horizontal axis.

The Sephadex particles were centrifuged for 5 minutes at 1000 × *g* and washed three times with 2 ml saline. After the last centrifugation, the pellet was resuspended in drop of saline and its radioactivity was measured in Nuclear Chicago counter.

In the main experimental series, the background non-specific adsorption of labelled ACTH to the Sephadex particles was not disturbing. In some experiments performed 18 months later it was found to vary with different batches of anti-IgE Sephadex, some batches giving considerable background absorption. Pre-incubation of the gel with low molecular protein, preferably thyrotropin (Actyron® see Results) reduced the non-specific adsorption of ACTH in such cases.

Calculations

The amount of labelled ACTH attached to the anti-IgE-Sephadex complex was determined from standard curves plotting log counts per minute (cpm) against log pg of ACTH. This relationship was approximately linear. The calculation was pursued on the basis of the following assumptions:

Mol w	Tetracosactid	2933.49
	IgE	196000.00

Combining proportions IgE = 2 ACTH.

The formula used for calculation was therefore the following:

$$\text{pg IgG} = \frac{\text{pg Tetracosactid}}{2 \text{ Mol w Tetracosactid}} \text{ Mol.w IgE.}$$

The pg IgE figure in the formula divided by 100 was called one unit (U). One unit thus came to correspond to about 1 ng. The applied formula could therefore be simplified to

Units IgE/ml = ng IgE/ml = 0.3341 pg Tetracosactid.

RAST Test for the Estimation of IgE Anti-ACTH (23)

ACTH was coupled to cyanogen bromide activated Sepharose as described by the manufacturer (Pharmacia Ltd., Uppsala, Sweden). 0.5 ml of the ACTH coupled gel containing 1 mg of gel/ml was incubated with 0.5 ml of the unknown sample for 18 hours at room temperature under vertical rotation of the test tubes. After separation of the test serum and three washings with 2 ml 0.15 M saline with 1 per cent Tween 20 the gel had 0.1 ml ^{125}I labelled anti-IgE (Pharmacia Ltd., Uppsala, Sweden) with a scintillation activity of about 40000 cpm added to it and incubation and washings were repeated as described above.

The radioactivity of the gel was then measured. Counts twice that of the negative control were taken to indicate specific reaction.

Chromato-electrophoresis

This test was performed as described by *Landon et al.* (12, 13) with the exception that serum was used instead of plasma. 0.2 ml of serum diluted 1:2 in 0.025 M phosphate buffer pH 7.5 containing human serum albumin 5 mg/ml and mercaptoethanol 0.5 per cent and 120 pg ^{131}I-tetracosactid in 0.2 ml of the same buffer were incubated for 4 days at +4 C. 0.1 ml was then removed immediately for the electrophoresis. The remainder was first acidified to pH 2.0 by 1 N HCl for 10 minutes.

The electrophoresis (13) was performed on Whatman No. 3 MC filter paper 37 × 55 mm using a barbital buffer pH 8.6 µ = 0.1. The runs were performed for 10 hours at 220 V and 3 mA/strip of filter paper.

The protein reactions were identified by staining with naphtalene black. The strips were divided into 0.5 cm pieces the radiation activity of which was measured in a Nuclear Chicago γ counter.

Absorption of Sera

Absorption was performed by incubating one volume of undiluted serum with one volume of packed BDB treated erythrocytes coated with the antigen for 1 hour at 37 C and 1 hour at 20° C.

TABLE 1 a *Reactivity against Synacthén® Homactid® and Postacton® in Three Diagnostic Groups Comprising 29 Asthma Patients 21 Rheumatic Patients and 50 Normal Subjects Respectively The Differences between the Individual Groups of Patients and the Normal Group Were Estimated Statistically*

Diagnostic group	Number of cases examined	Reaction against Synacthén Reactivity rates[1] %	P[2]	Homactid Reactivity rates[1] %	P[2]	Postacton Reactivity rates[1] %	P[2]
Asthma patients	29	75 4	<0 001***	48.3	>0 01	65.5	<0 001***
Rheumatic patients	21	84 0	<0 001**	80.8	<0 001***	71.6	<0 001***
Normal males	19	52 6	–	60 0	–	38.5	–
Normal females	31	48 4	–	60 0	–	29 7	–
Normal subjects (both sexes)	50	48 0	–	60.0	–	33.3	–

[1]) Compared at the titre level ≥ 1/10
[2]) P = statistical significance of difference between patient group and normal group. Highly significant values are marked by ***

Statistical Methods

Differences between reactivity rates were evaluated by the χ^2 test.

RESULTS

A *Agglutinating Antibodies Examined by the Passive Haemagglutination Test*

1 *Reactivity in diagnostic groups (Tables 1 a and b)* The reactions against Synacthén® Homactid® Postacton® porcine bovine and ovine γ-globulin were examined in the Acton® treated 29 asthmatic and 28 rheumatic patients who were resistant to ACTH therapy and in the 63 untreated persons without known disease. Sera from the 20 asthmatic patients more closely studied were obtained 12 months after the onset of an intensive course of ACTH therapy (see Material and Methods and 16) The groups were compared on the basis of their reactivity rates at the dilution 1/10 There were no obvious differences between male and female normal sera They were therefore treated as one group

The reactivity rates against Synacthén® Homactid® and Postacton® were higher among asthmatic and rheumatic patients than among normal subjects. It applies to all three substances that the difference between rheumatic and normal subjects was statistically significant The difference between asthmatic and normal subjects was statistically significant in the case of Synacthén® and Postacton® but not in the case of Homactid® (Table 1 a) It applies to both groups of patients that the reactivity rate against porcine γ-globulin but not that against bovine or ovine γ-globulin was significantly higher than that in the normal group (Table 1 b) Unexpectedly the rheumatic group showed a lowered reactivity rate against ovine γ globulin (Table 1 b)

2 *Distinction between normal and pathological agglutination reactions* By the aid of the curves reproduced in Fig 1 (see also Methods page 354) the distinctive titre level between pathological and normal reactions against Synacthén® Homactid® and Postacton® was set at 1/200 The reactivity rates at this titre level in the examined groups are shown in Table 2

Most pathological titres were moderate (Fig 1) Only one serum obtained from an

TABLE 1 b. *Reactivity against Bovine O ine and Porcine γ-Globulin in the Same Three Diagnostic Groups as in Table 1 a. The Differences between the Individual Groups f Patients and the Normal Groups Wer Estimated Statistically as in T ble 1 a.*

Diagnostic group	Number of cases examined	Reaction against					
		Bovine γ-glob.		Ovine γ-glob.		Porcine γ-glob.	
		Reactivity rates[1] %	P[2])	Reactivity rates) %	P[2])	Reactivity rates[1]) %	P[2])
Asthma patients	28	50 0	1.0	57.9	>0.99	29.6	<0.001***
Rheumatic patients	19	47.4	>0 7	58.5	<0.001***	58.5	<0 001***
Normal subjects (both sexes)	16	50.0	–	58.3	–	10.0	–

) Compared at the titre level 1/10

) P = statistical significance of difference between patients group and normal group. Highly significant values are marked by ***

asthma patient had a very high titre against *all* three antigens.

3 *Correlated antibody specificities.* As could be expected, the reactions against Synacthén® Homactid® and Acton® were correlated. However several sera reacted only with two of these antigens. Reactions against porcine γ-globulin were correlated not only with re actions against Acton but also with reactions against Synacthén® A correlation between the reactions against the synthetic ACTH preparations and the synthetic vasopressin *preparation, Postacton*® was also noted. Sta tistical evaluations of some of these cross reactivities have been accounted for in Table 3

Three types of correlated reactivity be-

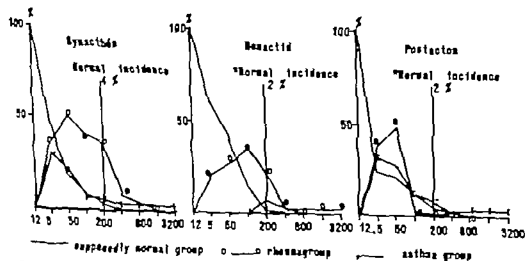


Fig 1 Incidence (per cent) distribution of reactions against Synacthén® Homactid® and Postacton® over twofold dilution series with the base 1/12.5 Distributions of sera from groups of rheumatic (28) asthmatic (29) and supposedly normal subjects (63) hav been reproduced.

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Rheumatic patients	21	84 0	<0.001**	80 8	<0.001**	71.6	<0.001***
Normal males	19	52.6	–	60 0	–	38.5	
Normal females	31	48.4	–	60 0	–	29 7	–
Normal subjects (both sexes)	50	48.0	–	60 0	–	33.3	–

1) Compared at the titre level ≥ 1/10

2) P = statistical significance of difference between patient group and normal group. Highly significant values are marked by ***

Statistical Methods

Differences between reactivity rates were evaluated by the χ^2 test.

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TABLE 1 b *Reactivity against Bovine Ovine and Porcine γ-Globulin in the Same Three Diagnostic Groups as in Table 1 a. The Differences between the Individual Group / Patient and the Normal Groups Were Estimated Statistically as in Table 1 a.*

Diagnostic group	Number of cases examined	Bovine γ-glob. Reactivity rates[1] %	Bovine γ-glob. P[2]	Ovine γ-glob. Reactivity rates[1] %	Ovine γ-glob. P[2]	Porcine γ-glob. Reactivity rates[1] %	Porcine γ-glob. P[2]
Asthma patients	28	50 0	1.0	57.9	>0.99	29.6	<0.001***
Rheumatic patients	19	47 4	>0.7	58.5	<0 001***	58.3	<0.001***
Normal subjects (both sexes)	16	50.0	–	58.3	–	10.0	–

[1]) Compared at the titre level 1/10.
[2]) P = statistical significance of difference between patients group and normal group. Highly significant values are marked by ***

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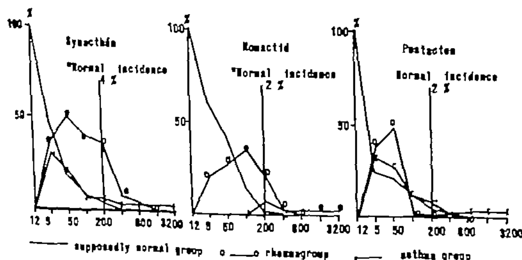


Fig 1 Incidence (per cent) distribution of reactions against Synacthén® Homactid® and Postacton® over twofold dilution series with the base 1/12 5. Distributions of sera from groups of rheumatic (28) asthmatic (29) and supposedly normal subjects (63) have been reproduced.

TABLE 2 *Reactivity Rates against Synacthén® Homactid® and Postacton® at the Adopted Dilution Level 1/?00 Obtained in Groups of Asthma Patients Rheumatoid Patients and Normal Persons*

Antigen	Asthma patients %	Rheumatoid patients %	Normal persons %
Synacthén®	10	40	4
Homactid®	10	?3	2
Postacton®	14	4	5

TABLE 3 *Statistically Significant Correlations between Reactions against some Tested Antigens*

Compared antigens	Regression coefficient	P for linearity of regression	Correlation coefficient	P for correlation coefficient	Number included in computation
Synacthén®/Homactid®	0.55	0 001	0 66	0 001	88
Synacthén®/Acton®	0 84	0 005	0.95	0 001	7
Synacthén®/Postacton®	0 41	0 001	0 41	0 001	74
Synacthén®/Porc. γ glob	0 36	0 01	0 41	0 01	44

tween Synacthén® and Postacton® could be distinguished by haemagglutination inhibition and absorption tests. In one case, the mixed haemadsorption reactions against Synacthén® and Postacton® were mutually inhibited by either antigen (10) Usually however crosswise inhibition or crosswise absorption with antigen coated erythrocytes was not successful. The agglutination reactions of one serum against Synacthén® and Postacton® coated on BDB treated erythrocytes was not inhibited by either homologous or heterologous antigen added as a solution Nor did the serum react against Synacthén® or Postacton® in the mixed haemadsorption test. The reacting antibodies could be absorbed, however by *erythrocytes* coated with either homologous or the heterologous antigen Cells coated with several other protein antigens such as porcine bovine and ovine γ-globulin were ineffective in this respect

B *IgE-determinations*

1 *Methodological experiments* The Ig class specificity of the method employed was roughly evaluated by comparing the contents of IgE anti ACTH measured in some heated and unheated sera (Table 4) Heating was found to reduce the specific antibody contents

TABLE 4 *Effect of Heating of Se a from some Asthmatic and Normal Subjects on the Measured Contents in Sera of IgE Anti-ACTH*

Clinical group	Serum prototype number	Contents of IgE anti-ACTH units/ml in	
		Untreated serum	Heated serum
Asthma patients	W 1009 3	84	30
	W 1010 1	101	51
	W 1017 3	64	17
Normal subjects	N 6	40	16
	N 148	15	11

TABLE 3 *Effect on the Measured Contents of IgE Anti-ACTH in the Sera of some Asthmatics Produced by Adding Synacthén® to the Mixt re f Reactants*

Clinical group	Serum prototype number	Measured IgE contents units/ml			
		1st experiment		2nd experiment	
		Without Synacthén®	Synacthén® added	Without Synacthén®	Synacthén® added
Asthma patients	W 1002.5	142.5	28.4	114.5	10 6
	W 1010 1	100.9	12.8	88.9	50.0
	W 1017.5	81 1	33 7	53.0	11.9

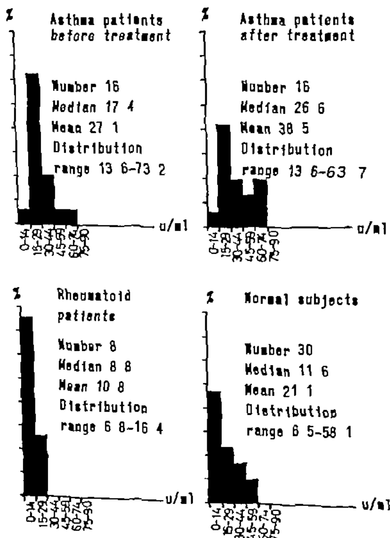


Fig 2 Concentration distribution of IgE-anti-ACTH as measured by modified RAST-technique in sera from groups of asthmatic and rheumatic patients and in group of normal subjects.

TABLE 6 *Degree of Non-specific Absorption of ¹²⁵I Conjugated ACTH on Two Different Lots of Sephadex Anti IgE Gel and the Blocking Effect of Different Low-molecular Proteins on the Degree of ACTH Absorption to the Highly Absorbing Lot*

Sephadex gel	Blocking substance	Background activity
"Non-absorbing" lot	Unblocked	789
Highly absorbing lot	Unblocked	11509
	Actyron®	1224
	HSA	2120
	Insulin	2279
	HGG	2762

of one patient's serum by about 50 units while two sera from blood donors showed a reduction of 24 and 4 units, respectively

The serological specificity of the method was examined by adding Synacthén® to the system (Table 5) This was found to reduce the measured specific antibody contents by 50–100 units.

The reproducibility of the method was chiefly influenced by the varying degrees of background adsorption to be noted when different lots of anti IgE coupled Sephadex gel were used (Table 6) This cause of variation was disturbing in some lots of gel It could be partly assessed by preincubation with an antigenically indifferent, low molecular protein Actyron® (thyrotropin) being the most effective among the substances tested (Table 6)

2. *Reactivity of diagnostic groups* Specific IgE anti-ACTH antibodies were determined in most of the sera using the modified radio-immunosorbent test. The concentration of such antibodies measured tentatively in units ranged between 1 and 58 u/ml in 30 apparently healthy persons, a mean of 21 u/ml and a median of 11 6 u/ml (Fig 2)

In 16 asthma patients the median was 17 4 and the mean 27 1 u/ml. During treatment, these parameters rose to 26 7 and 38.5 u/ml respectively Both the median and the mean concentrations were in 8 rheumatoid patients distinctly below the corresponding figures in normal individuals (Fig 2, Table 7)

3 *RAST tests for IgE anti ACTH* After the establishment of the cyanogen bromide coupled antigen RAST test as a routine procedure, a number of the sera reacting in the previously described radio-immuno assay were tested in the RAST test as well. It applies to most sera that the noted fluctuation of the cpm was within the accepted range of variation among normal subjects. Thus, the standard version of this technique could not provide information other than that obtained by the modified radio-immuno assay described in the previous section

C. Other Methods

1 *Mixed haemadsorption tests* Although it soon became evident that the passive haemagglutination technique using BDB conjugated antigen was preferable to the mixed haemadsorption test for ACTH antibodies, a limited study using the latter technique, was performed The aim was to find some reacting sera to be used in the development of the passive haemagglutination test and to make sure that non-agglutinating antibodies were not present in many sera.

The difference between sera from normal subjects and sera from patients was usually small. However sera from some patients attained considerable titres.

As expected, there was correlation although not complete, between the mixed haemadsorption technique and the passive haemagglutination technique. It applies to

TABLE 7 *Reactivity Rate of IgE-anti-ACTH against ¹²⁵I-Acth in Groups of Asthma Patients Rheumatic Patients and Normal Subjects*

Diagnostic group	Reactivity rate at 20 u/ml level	P for difference from normal*
Asthma patients (on routine treatment)	8/16	0 7
Rheumatoid patients (on routine treatment)	0/8	<0 001
Normal subjects	10/30	–

* P estimated by the χ² test.

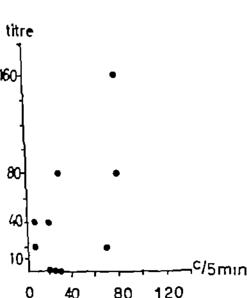


Fig. 3. Correlation between titres against Synacthén® in agglutination test and γ-counts from ACTH-^{125}I in γ-globulin area after paper electrophoresis of serum incubated with the labelled ACTH.

the former as well as to the latter technique that there was an obvious correlation between the reactions against the three hormonal substances tested (Synacthén® Hormactid® Portacton®). Antibodies reacting with only ACTH or vasopressin, respectively, were noted.

2. *Chromatoelectrophoresis.* A small number of sera were examined by chromatoelectrophoresis according to *Landon et al.* (12, 13). An association between the γ counts in the IgG electrophoretic fractions and the passive haemagglutination titre was noted (Fig. 3).

DISCUSSION

The results which are in agreement with those obtained in several earlier investigations (9, 17, 18, 21) show that treatment with ACTH preparations involves the risk of immunization against the hormone part of these peptides. This is a biologically remarkable fact since it implies that antibodies are produced against a protein configuration that is probably common to the entire vertebrate series and engaged in vital functions. In this respect the ACTH antibodies resemble the DNA antibodies.

Of course, specific antibodies are produced, however, when immunization occurs in response to treatment with non human pituitary extracts. An example of this is the increased incidence of antibodies against porcine gamma globulin both in the asthmatic group and in the rheumatic group. The reactions were correlated to those against ACTH, but reactions against either antigen might also occur independently.

The agglutination titres against Synacthén® Hormactid® and Portacton® were usually somewhat higher in Acton treated cases than in Synacthén® treated cases. This may be due to an adjuvant effect of the species part of the Acton molecule. A corresponding difference in the IgE response was not noted. Since all patients had been treated with Acton on some previous occasion, it is not possible to decide on the basis of the present results whether tetracosactid or octacosactid *per se* can be primary immunogens or whether they can act only as "booster" antigens in patients previously immunized by natural extracts.

Thus, the full extent of the immune response to ACTH cannot be fully evaluated at present. However, it is apparent that IgG and IgE responses may vary independently and that asthma patients are more inclined to react by IgE antibodies while rheumatic patients more readily react by IgM IgG antibodies. The engagement of the cellular immunity remains to be explored.

Reactions against Synacthén® and Hormactid® were closely but not completely correlated, implying that the structural difference between these substances (tetra- and octacosactid, respectively) sometimes is decisive for their immunogenicity. For this reason, a change from therapy with one substance to the other may be of clinical benefit in some instances.

Rather unexpectedly it was found that most sera reacting with ACTH reacted also with vasopressin. Evidence pointing in this direction has also been noted by *Mulder* (personal

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P estimated by the χ^2 test.

Chromato-electrophoresis examinations were performed in order to control the results of the agglutination technique by an independent method. Although only a few sera were compared, the results obtained by the two methods were tolerably correlated

The clinical importance of immunization against ACTH preparations has been emphasized in a separate report (16) The principal clinical symptoms have been either anaphylactic reactions (9 16, 25) or refractoriness to *ACTH therapy (10) Refractoriness to ACTH* which is most probably due to the blocking of the hormone active site with specifically reacting IgG has, to the authors knowledge, been noted predominantly in rheumatic subjects. Serious anaphylactic responses to administered ACTH may not occur when the ACTH specific antibodies consist of IgG in excess over IgE. The noted tendency of rheumatic subjects to form IgG in excess over IgE response to ACTH may explain the rarity of anaphylactic reactions to ACTH treatment in these cases.

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REFERENCES

1 *Arnoldsson H* Long-term Acth and corticosteroid therapy in bronchial asthma. Allergic reactions to Acth Acta Allerg Suppl. 6, Chapter XI, p 122 133 1958

2 *Baumann G Rayfield E J Rose L I Williams G H & Dingman J* Trace contamination of corticotropin and human growth hormone with vasopressin—clinical significance J Clin Endocr 34 801–804 1972

3 *Bek L & Holm S* Immunologiska studier med organiskt och syntetiskt ACTH Nord Med 74 677 1965 (In Swedish)

4 *Boyden S V* The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera J exp Med 93 107 120, 1951

5 *Espmark A Biberfeld G F graeus A*

30

Johansen T., Jonsson J & Magnusson B., Virological findings during the smallpox outbreak in Stockholm in 1963 Acta Med. Scand. Suppl. 464 1966.

6. *Fleischer N., Abe K., Liddle G W., Orth D N & Nicolson W E.*, ACTH antibodies in patients receiving depot porcine ACTH to hasten recovery from pituitary adrenal suppression. J Clin. Invest. *46* 196–204 1967

7 *Forsman O Kerrgren M., Nerd H & Paulsen F.:* Allergy to Acth of animal and human origin. Acta Allerg *18* 462–470 1963

8. *Ishizaka K., Ishizaka, T & Hornbrook, M.*, Allergen-binding activity of γE, γG and γA antibodies in sera from atopic patients. J Immunol. *98* 490–501 1967

9 *Jensen N E & Sneddon I.*, Allergic intolerance to tetracosactrin. Brit. Med. J *1* 583–584 1969

10. *Jonsson J Hasermark G & Olhagen B.*, Auto-antibodies to pituitary tissue and pituitary hormones in case of systemic lupus erythematosus. Acta Rheum. Scand. *15* 21–28, 1969

11 *Jonsson, J* Application of mixed haemadsorption—a mixed antiglobulin reaction—to soluble antigens fixed on glass slides. Acta path. microbiol. scand. Suppl 202, 1969

12. *Landon J., Livanou Th. & Greenwood F E.*, The preparation and immunological properties of [131]I-labelled adrenocorticotrophin. Biochem. J *105* 1075–1083 1967

13 *Landon J., Friedman M & Greenwood F C* Antibodies to corticotrophin and their relation to adrenal function in children receiving corticotrophin therapy Lancet *i* 652–655 1967

14 *Loren I. Arse B. S & Aise, S* The immunological properties of human Acth. Acta Endocr Suppl. 89 12, 1964

15 *Meeder E. & Schwarz-Speck M* Allergitestemit natürlichem und synthetischem ACTH. Schweiz. Ges. Derm. Vener 45 Jahresvers. Basel 1963 Dermatologica 129 59–70 1964

16. *Pelow K-O & Jonsson J* Immune responses to treatment with natural and synthetic ACTH in bronchial asthma. Acta Allergologica *30* 339–362, 1975

17 *Ratcliffe J G Pritchard M & El Shaboury A H* The production of antibodies to porcine corticotrophin and to Synacthén. Proc Soc. for Endocr. Endocrinol. *43* 1–11, 1969

18 *Serafini U.*, Long-term therapy of asthma with hormones. Acta Allerg Suppl. 7 263–274 1960.

19 *Serafini U., Torrigiani, G & Masala C.*, ACTH antibodies. I *Polvani F* and *Crosignani, P* (Eds.) Immunological properties of protein hormones New York and London 1966 p. 15–28

communication) The nature of this reactivity is apparently complex. A mutual haemagglutination inhibition effect on a few sera indicates that identical determinants occasionally may be shared by the two antigens. In one case a high titre reactivity to the two synthetic hormones appeared only when the latter were BDB-conjugated to sheep erythrocytes. The cause of this unusual finding remains to be reliably assessed In most instances, however apparently different antibodies were reacting to different parts of the two molecules This suggests that the associations between reactions against ACTH and vasopressin are most commonly due to an otherwise undetectable contamination of the natural ACTH with vasopressin (2) The fact that antibodies to vasopressin can be elicited by 'booster' injections of tetracosactid may represent a kind of coupled cathamnestic response It is rather remarkable however that a cathamnestic response usually was not elicited at the same time against Acton® Synacthén® or porcine γ-globulin

The experiments performed in the present study have provided some methodological experience

For the present purpose the mixed haemadsorption technique was inferior to the passive haemagglutination technique with respect to reproducibility and freedom from non specific background reactions. The tanned red cell technique was successful in the case of some antigens (Acton Postacton the gamma globulins) while the tetra and octacosactids required conjugation with bis-diazotized benzidine. It applies also to vasopressin (Postacton®) that the latter technique was preferable. The possible usefulness of cells treated with chromic acid was not tested in this study

The principle of the immuno-assay employed for determining IgE anti ACTH was that originally devised by Ishizaka et al (8) i e. an excess of radio-labelled antigen was added to sera containing IgE antibody The IgE-antigen complexes were collected by attaching them to anti human IgE. In the present case, the anti-IgE was conjugated to Sephadex particles (23) A comparable solid phase radio-immuno-assay has recently been described by Zeiss et al (24) In the choice of reagents, advantage could be taken of the fact that some well-defined and controlled reagents used in the RIST test were commercially available. Non-specific adsorption of ACTH to the Sephadex gel was a potential source of error but could be kept at a tolerable level by choosing low-absorbing gel batches. If needed, the nonspecific adsorption could be much reduced by pre-incubating the gel with Actyron® (thyrotropin) As the RAST technique became available at a relatively late stage of the study, many of the sera were retested by this technique. However the observed variation between the sera was too small to be of statistical significance. The first employed technique was therefore used throughout although a modified, more sensitive RAST might have served the same purpose

The concentration of IgE anti ACTH measured is low in all three groups examined which testifies to the weak immunogenicity of tetracosactid Nevertheless, there was a distinct difference between the reactivity rates in asthmatics and in rheumatics and this difference was statistically significant.

The measured ACTH uptake obtained with normal sera was recorded as if it meant the presence of a certain low IgE anti ACTH concentration in these sera However the concentration is most probably spurious and due to a nonspecific adsorption of ^{125}I ACTH to the Sephadex gel. Accordingly it might equally well have been subtracted from the measured counts. However in the situation at hand it was thought of advantage for the statistical evaluation and the clarity of presentation to fix a level of measured antibody concentration to constitute a supposed border level between pathological and normal concentrations. Besides, if antibodies are considered from the point of view of clinical diagnosis it might often be more cautious if the magnitude of the range between normal and pathological were evaluated rather than to decide between existence and non-existence (22)

STUDIES OF SMOOTH-MUSCLE ANTIBODIES IN ACUTE HEPATITIS

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Andersen, P., Thestrup-Pedersen, K. & Ladefoged, K. Studies on smooth-muscle antibodies in acute hepatitis. Acta path. microbiol. scand. Sect. C, 84 365–371 1976.

Smooth-muscle antibodies (SMA) of the IgG and IgM class in titres of 10 to 60 were demonstrated in 19 of 38 patients with acute hepatitis (50 per cent). No IgA-SMA were detected. IgM-SMA occurred in the beginning of the disease, while IgG-SMA were found both early and late in the course of disease. There was no correlation between the occurrence of SMA and the biochemical liver parameters or the mode of exposure to infection, but IgM-SMA were found more often in the first serum sample obtained from patients without hepatitis-B-associated antigen (HBAg) than in HBAg-positive patients ($p_1 + p_2 = 0.04$) and it is suggested that hepatitis virus A more readily than hepatitis virus B can elicit the formation of SMA. The mean PHA-induced lymphocyte-transformation response was significantly lower in hepatitis patients than in the controls ($0.02 > p > 0.01$). The lymphocyte-transformation response to a low PHA dose (1 µg per ml) was lower in hepatitis patients without SMA than in those with SMA ($0.10 > p > 0.05$). SMA were not found in 28 control persons, and the incidence of tissue antibodies other than SMA did not differ in hepatitis patients and controls.

Key words: Smooth-muscle antibodies; acute hepatitis.

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Human antibodies reactive with smooth-muscle actomyosin have been detected in serum from patients with acute infective hepatitis (2, 9, 15, 19), infectious mononucleosis (11, 16) and cytomegalovirus infection (5). The reason why smooth-muscle antibodies (SMA) arise in viral infections is not clear and we have therefore studied some of the factors which might influence the development of SMA in acute hepatitis.

Liver cell damage has been suggested to be a direct stimulus for SMA production in acute hepatitis (9, 10), therefore the development of SMA was studied in relation to the liver function judged by biochemical liver parameters. It was also studied whether hepatitis-B-associated antigen (HBAg) and the mode of exposure to infection were related to the occurrence of SMA, as the production of this antibody may differ in different forms of acute hepatitis. Several authors have shown that the phytohaemagglutinin (PHA) response of lymphocytes from patients with acute hepatitis is impaired (1, 13, 14, 18). However, the influence of the impaired T-cell function in the production of SMA has not been studied earlier. Therefore we have investigated the T-cell function by the reactivity of lymphocytes to stimulation with

20 *Staritsky A B & Arquilla E. R.*, Studies of proteins and antibodies by specific hemagglutination and hemolysis of protein conjugated erythrocytes. Int. Arch. Allergy *13* 1–36 1958

21 *West H F* Ten years of Acth therapy Ann. Rheum. Dis *21* 263–271 1962

22 *Werner M Biberfeld G Fagraeus A & Jonsson J* Autoantibodies in healthy persons. Unpublished results.

23 *Wide L.* Solid phase antigen-antibody systems. In *Kirkham K E* and *Hunter W M* (Eds.) Radioimmunoassay Methods. Churchill Livingstone Edinburgh and London 1971 p 405–412

24 *Zeiss R C Pruzansky J J Patterson R. & Roberts M* A solid phase radioimmunoassay for the quantitation of human reaginic antibody against ragweed antigen E. J Immunol. *110* 414–421 1973

25 *Abjörn Ch & Leonhardt T* Anafylaktisk chock med dödlig utgång efter injektion av syntetiskt kortikotropin (Synacthén R Depot) hos patient med svår asthma. Sv. Läkartidn. *67* 4364–4366 1970 (In Swedish)

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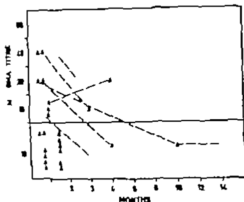


Fig. 1. IgM-SMA in 66 sera from 38 patients with acute hepatitis, related to the course of disease. Solid symbols represent serum samples with hepatitis-B-associated antigen (HBAg) and open symbols HBAg-negative sera.

of 28 control persons. SMA were of the IgM class in 10 cases and of the IgG class in six cases, while three patients had both IgG and IgM antibodies in serum. IgA-SMA could not be demonstrated in any of the patients.

IgM-SMA were present most often and in the highest titres in the beginning of disease (Fig. 1). In five patients, the IgM-SMA titre decreased during the observation period and the titre increased only in one case. The latter was the only case in which IgM-SMA could be demonstrated more than 3 months after the onset of disease.

IgG-SMA were present in the first serum sample obtained within 2 months after the onset of disease in seven patients, in two other patients the antibodies appeared later (Fig. 2). Two or three serum samples were obtained from seven of the nine IgG-SMA positive patients. In three of these patients, IgG-SMA disappeared from serum (i.e. titre below 10), while the IgG-SMA titre increased in the remaining four. The antibodies could still be detected in serum from these four patients 3½ to 14 months after the onset of disease. Thus, IgG-SMA persisted in serum for a longer period of time than IgM-SMA.

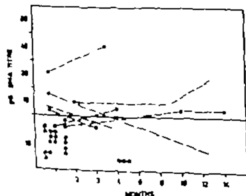


Fig. 2. IgG-SMA in 66 sera from 38 patients with acute hepatitis, related to the course of disease. Solid circles represent serum samples with hepatitis-B-associated antigen (HBAg) and open circles HBAg-negative sera.

SMA in Relation to Biochemical Liver Parameters

The relationship between the maximum values of S-GOT and serum bilirubin during the course of disease as well as the values at the time of the serological investigation were studied in relation to the SMA-titres. However, no correlation between the S-GOT values or the serum-bilirubin concentrations and the SMA titres was demonstrated.

The liver function was abnormal after 3½ and 4 months in two patients in whom the S-GOT values and the gamma-globulin concentration were still elevated. In both cases, IgG-SMA persisted in serum in titres of 10 and 40 respectively and IgM-SMA were found in one of these in a titre of 10. In the remaining 20 patients who were examined two or three times, including two with IgM-SMA and two with IgG-SMA, the S-GOT values were below 30 units and the serum-bilirubin concentration below 1.5 mg per 100 ml at the time of the second investigation.

HBAg and Mode of Exposure to Infection

HBAg was demonstrated in the first serum sample from 15 of 38 patients with acute hepatitis (39 per cent). HBAg was found in 11 out of 22 (50 per cent) parenterally infected patients and in 4 out of 16 (25 per cent) non-parenterally infected patients (Fisher's

PHA and studied whether alterations in the T-cell function might influence the production of SMA in acute hepatitis.

MATERIAL AND METHODS

Subjects

Fourteen women and 24 men, aged from 16 to 64 years, with acute hepatitis were examined. A liver biopsy was obtained from all patients and the diagnosis of acute hepatitis was based on clinical biochemical and histopathological findings (8). The microscopic examination of liver biopsies was performed at the Department of Pathology, Randers City Hospital. Thirty two patients were examined within 1½ months after onset of symptoms and six were examined more than 1½ months after the onset. In addition to the first investigation, 22 patients were studied again once or twice from 2½ months to 15 months after the onset of disease. PHA stimulation of lymphocytes was only investigated in 20 patients. According to the mode of exposure to infection the patients were classified into two groups, one comprising parenterally infected patients, the other the non parenterally infected patients. The group of parenterally infected patients comprised seven women and 15 men who had received injections, blood transfusions or they had been tatooed within a 6-month-period preceding onset of disease. Eighteen of these patients were drug addicts. The group of non parenterally infected patients comprised seven women and nine men without any history of injections.

A control group consisting of 15 women and 13 men aged from 23 to 71 years was also investigated. None of the controls had auto-immune infectious or hepatic disease. They were HBAg negative and their biochemical liver parameters were normal. PHA-induced stimulation of lymphocytes was only investigated in eight of the controls. Therefore 14 normal men who had participated in a study of infertility were included in the control group for PHA stimulation. They were examined during the same period of time and by the same culture technique as the other control and patients. These men were all healthy but liver function tests were not performed.

Tissue Antibodies

A total of 94 sera from patients with acute hepatitis and normal persons was investigated for smooth muscle antibodies (SMA), glomerular antibodies (GA), antinuclear antibodies (ANA), parietal-cell antibodies (PA) and mitochondrial antibodies (MTA) by means of the indirect immunofluorescent method (IIF) as described earlier (3, 4). The substrates were 4 μm thick cryostat sections of rat stomach and rat kidney. All sera were investigated for antibodies of the IgG, IgA and IgM classes by means of monospecific FITC-labelled conjugates (Wellcome Research Laboratories, England). The molar F/P ratio of the conjugates ranged from 2.9 to 3.5 and all conjugates were used in a dilution corresponding to an antibody content of 1·4 to 1·8 units per ml (6). The sera were initially tested in a dilution of 1:10, and all positive or doubtfully positive sera were titrated in doubling dilution starting at 1:10. All sera from one patient were investigated in the same experiment.

Hepatitis-B-associated Antigen

Hepatitis-B-associated antigen (HBAg) was demonstrated by a radioimmunoassay (AUSRIA II). The reactions were kindly performed by Dr. *J. Jørgensen, the Blood Bank, Aarhus Municipal Hospital.*

Biochemical Liver Tests

The serum bilirubin concentration, the serum glutamic oxaloacetic transaminase (S-GOT) activity and the serum gamma-globulin concentration were determined by routine methods at the Department of Clinical Chemistry, Randers City Hospital.

Lymphocyte-transformation Test

Approximately 20 ml of venous blood was drawn into a test tube containing phenol-free heparin to produce a final concentration of 20 i.u. of heparin per ml. The mononuclear cells were isolated on a Ficoll-Isopaque gradient and washed twice in TC-199 with 5 per cent pooled inactivated A serum and 20 i.u. of heparin per ml. A cell suspension of 0.5×10^6 lymphocytes per ml was prepared and portions of 2 ml of this suspension were placed in culture tubes. Phytohaemagglutinin (PHA P Wellcome MR68) was added to give a concentration of 1 μg per ml or 3 μg per ml. Nothing was added to the control cultures. The cultures were made in triplicate and incubated at 37 C. The duration of culture was 3 days. 24 hours before harvesting, 0.2 μCi of ^{14}C-labelled thymidine was added. The cells were harvested on Whatman glass-fibre filters (GF/B2) washed in saline and alcohol, and counting was made in a scintillation counter (Packard Tri-carb). The results are expressed as disintegrations per minute after correction for quenching and efficiency.

RESULTS

The Occurrence of SMA in Serum

SMA in titres of 10 to 80 were found in 19 out of 38 patients with acute hepatitis (50 per cent), but could not be demonstrated in any

TABLE 2 *PHA-induced Lymphocyte Response in HBAg-positive and HBAg-negative Patients with Acute Hepatitis and in Controls*

	PHA 1 µg per ml dpm × 10⁻³		PHA 3 µg per ml dpm × 10⁻³	
	Mean	S.D	Mean	S.D
HBAg-positive patients with acute hepatitis	71.2 n = 8	39.8	86.8 n = 7	32.1
HBAg-negative patients with acute hepatitis	87.2 n = 12	43.9	92.9 n = 9	26.5
HBAg-positive and HBAg-negative patients with acute hepatitis	80.6 n = 20	42.0	90.3 n = 16	28.3
Controls	114.7 n = 22	40.0	N.D	

S.D standard deviation dpm, disintegrations per minute n, number of patients N.D., not done.

ml, the mean response was significantly lower in patients than in controls ($0.02>p>0.01$). The mean PHA response was lower in HBAg-positive patients than in HBAg negative patients, but this difference was not significant. The PHA induced lymphocyte transformation was found to be significantly lower in eight HBAg-positive patients than in 22 controls ($0.025>p>0.02$) while the difference in mean response of 12 HBAg-negative patients and the controls was not significant.

The correlation between the PHA-induced lymphocyte response and the occurrence of SMA in patients with acute infective hepatitis is shown in Table 3. Using PHA in a concentration of 1 µg per ml, the lymphocyte response would be higher in patients with SMA than in those without SMA, but this difference was not significant ($0.10>p>0.05$). In the SMA positive patients, SMA were of the IgM class in eight cases and of the IgG class in three. When PHA was used in a concentration of 3 µg per ml, the lymphocyte transformation response would be the same in SMA positive and SMA negative patients.

DISCUSSION

Farrow et al. (9) and *Ajdukiewicz et al.* (2) found SMA in 87 per cent and 74 per cent, respectively of patients with acute hepatitis.

TABLE 3 *PHA-induced Lymphocyte Response in SMA-positive and SMA-negative Patients with Acute Infective Hepatitis*

	PHA 1 µg per ml dpm × 10⁻³		PHA 3 µg per ml dpm × 10⁻³	
	Mean	S D	Mean	S D
SMA-positive patients with acute hepatitis	96.0 n = 11	43.9	92.1 n = 9	27.7
SMA-negative patients with acute hepatitis	62.5 n = 9	32.7	93.6 n = 5	34.5

S.D standard deviation dpm, disintegrations per minute n, number of patients.
SMA were of the IgM class in eight cases and of the IgG class in three cases.

TABLE 1 *The Occurrence of IgG- and IgM-SMA in the First Serum Sample in HBAg-positive and HBAg negative Patients with Acute Hepatitis and in HBAg-negative Controls*

	SMA positive			SMA-negative
	IgG and/or IgM	IgG	IgM	
HBAg positive patients with acute hepatitis (15 pts)	5* (33 %)	4 (27 %)	2 (13 %)	10 (67 %)
HBAg negative patients with acute hepatitis (23 pts)	13* (57 %)	3 (13 %)	11 (48 %)	10 (43 %)
HBAg negative controls (28 pers)	0	0	0	28

* One serum contained both IgG- and IgM-SMA.

exact test $p_1 = 0.11$) Eleven of the initially HBAg positive patients were examined later in 10 of these HBAg disappeared from serum within 2 months after the onset of disease, whereas it persisted in one patient 3¼ months after the onset (Fig 1) HBAg could not be detected at a later stage of the disease in any of the patients who initially had been HBAg negative.

SMA in Relation to HBAg and Mode of Exposure to Infection

It is seen from Table 1 that SMA were found in the first serum sample in five of 15 HBAg positive patients (33 per cent) and in 13 of 23 HBAg negative patients (57 per cent) SMA in titres above 10 were found in one of 15 HBAg positive patients (7 per cent) and in nine of 23 HBAg negative patients (39 per cent) this difference was statistically significant (Fisher's exact test $p_1 + p_2 = 0.031$) Thus, SMA in titres above 10 occurred more often in HBAg negative than in HBAg positive patients with acute hepatitis. IgG-SMA were found more often in the HBAg-positive (27 per cent) than in the HBAg negative patients (13 per cent) but this difference was not significant The rate of occurrence of IgM-SMA was significantly higher in the HBAg negative (48 per cent) than in the HBAg positive patients (13 per cent) (Fisher's exact test $p_1 = 0.03$ $p + p_2 = 0.04$) The IgM-SMA titres were higher in the HBAg negative than in HBAg positive patients as the IgM-SMA titres in the HBAg-positive patients did not exceed 10

Among 22 parenterally infected patients, SMA were found in 11 (50 per cent) and among 16 non parenterally infected patients, SMA were demonstrated in seven patients (44 per cent) This difference was not significant.

Other Tissue Antibodies

In patients with acute hepatitis, ANA of the IgM class were found in a titre of 10 in two patients in one of these, SMA were also present. GA were found in six sera, including five in which the SMA titre was the same as, or higher than the GA titre. The last serum contained IgM-GA in a titre of 40 but it did not contain SMA IgG PA in titres of 10 and 20 occurred in three patients who were all SMA negative

Among the control persons, IgG-ANA in a titre of 10 were found in one case IgM ANA in titres of 10 and 40 were present in two cases and IgG PA in a titre of 10 in one case. None of the persons had SMA in serum.

No MTA were demonstrated either in the hepatitis patients or in the controls.

PHA induced Lymphocyte Response

The results of the lymphocyte response to PHA stimulation in patients with acute hepatitis and in controls are shown in Table 2 Using PHA in a concentration of 1 µg per

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S D standard deviation dpm, disintegrations per minute n, number of patients.
SMA were of the IgM class in eight cases and of the IgG class in three cases.

The incidence of SMA was lower in our study probably because 15 of our 38 patients were not examined until more than 1 month after the onset of disease and SMA of the IgM class may have disappeared at this stage

It has been suggested that liver-cell damage may be the stimulus for SMA production (9 10) However if the liver-cell damage is reflected by the S-GOT activity in serum or by the serum-bilirubin concentration no correlation between the occurrence of SMA and liver-cell damage could be established in the present study Neither could any relationship between the occurrence of SMA and a prolonged course of disease be demonstrated It was not possible to establish any relationship between the mode of exposure to infection and the occurrence of SMA (9) However in the acute stage of disease IgM-SMA occurred more often in HBAg negative than in HBAg positive patients. Thus it could be that hepatitis virus A more readily than hepatitis virus B elicit the formation of these antibodies. This is in agreement with some previous investigations (14) and in contrast to others (9 19)

The present finding of a reduced PHA induced lymphocyte response in patients with acute hepatitis is in agreement with some previous studies (1 13 14 18) and in contrast to others (17) where the PHA response was found to be normal The differences in lymphocyte response to PHA may be explained by the concentration used in the cell cultures, as the response depends on the PHA dosage used

Some observations suggest that an impaired lymphocyte-transformation response may best be revealed if a suboptimal dose of PHA is used (12) This may explain why we found that the PHA induced lymphocyte response in hepatitis patients would be lower when the mitogen was used in a concentration of 1 μg per ml than when it was used in a concentration of 3 μg per ml Lymphocyte transformation to PHA has generally been regarded as a marker of the T-cell function, although some studies have shown that B lymphocytes may also respond to PHA (7) We found that when a low PHA concentration (i.e. 1 μg per ml) was used in the lymphocyte-transformation test response in patients without SMA would be lower than response in patients with these antibodies. As the number of patients studied was relatively small, it is difficult to draw any definite conclusions from these data However in cases where the infection can lead to suppression of the T-cell function, judged by the PHA response, it cannot be precluded that the infectious agent may less readily induce production of SMA.

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REFERENCES

1 *Agarwal S S Blumberg B. S., Gerstley Betty J S London W T Millman I Sutnick A I & Loeb L. S* Lymphocyte transformation and hepatitis. Proc. Soc. exp. Biol. (N Y) *137* 1498–1502 1971

2 *Ajdukiewicz A B Dudley F J Fox R A Doniach D & Sherlock S* Immunological studies in an epidemic of infective short incubation hepatitis. Lancet i 803–805 1972.

3 *Andersen P & Beutner E. H* Defined immunofluorescence studies of cytoplasmic antibodies. Int. Arch. Allergy *43* 780–790, 1972.

4 *Andersen P* Indirect immunofluorescence studies of smooth muscle antibodies. Acta path. microbiol scand. Sect. B, *8?* 577–584 1974

5 *Andersen P & Andersen H K* Smooth muscle antibodies and other tissue antibodies in cytomegalovirus infection Clin. exp. Immunol ?2 2?–29 1975

6 *Beutner E H Sepulveda M P & Barnett E. V* Quantitative studies of immunofluorescent staining Bull. Wld. Hlth. Org 39 587 606 1968

7 *Chess L. McDermott R. P & Schlossman S F* Immunologic functions of isolated human lymphocyte subpopulations. I Quantitative isolation of human T and B cells and response to mitogens. J Immunol *113* 1113–1121 1974

8 *Christoffersen P & Poulsen H* Leverbiopsi ved virushepatitis Ugeskr f læger *135* 107 112 1973

9 *Farrow L. J Holborow E. J Johnson G D Lamb S G Stewart J S Taylor P E. Zuckerman A J* Autoantibodies and the

hepatitis-associated antigen in acute infective hepatitis. Br Med. J 2 693–695 1970.

10. *Farrow L. J Holborow E. J & Brighton, W D* Reaction of human smooth muscle antibody with liver cells. Nature new Biol. 232 186–187 1971

11 *Holborow E. J., Hemsted E. H & M ad S V* Smooth muscle autoantibodies in infectious mononucleosis. Br Med. J 3 323–325 1973

12. *Hosking, C. S Fitzgerald M G & Simons M J* Quantified deficiency of lymphocyte response to phytohaemagglutinin in immune deficiency diseases. Clin. exp Immunol. 9 467–476, 1971

13. *Mertens, G A Rössler R Havemann, K & Dölle W* Inhibited lymphocytic response to phytohaemagglutinin in acute infectious hepatitis and in chronic aggressive hepatitis indirect evidence for virus persistence in chronic aggressive hepatitis? Scand J Gastroent. suppl 7 39–46, 1970.

14 *Newble D I Holmes K T Wangel A. G & Forbes I J* Immune reactions in acute virus hepatitis. Clin. exp. Immunol. *20* 17–28, 1975.

15 *Sterner G Biberfeld Gunnel Landmark C & Norberg, R.* Kliniska och serologiska aspekter på kronisk aktiv hepatit. Nord. Med. *86* 932–933 1971

16. *Sutton R. N P Emond R T D Thomas D B & Doniach D.,* The occurrence of autoantibodies in infectious mononucleosis. Clin. exp Immunol. *17* 427–436 1974

17 *Tong, J M., Wallac A. M., Peters R. L. & Reynolds T B* Lymphocyte stimulation in hepatitis B infections. New Engl. J Med. 293 318–322, 1975

18 *Willems F Th. C Melnick J L. & Rawls W E.,* Viral inhibition of the phytohemagglutinin response of human lymphocytes and application t viral hepatitis. Proc. Soc. exp. Biol. (N Y) *130* 652–661 1969

19 *Wright R.* Australia antigen and smooth-muscle antibody in acute and chronic hepatitis. Lancet *i* 521 1970.

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THE SEROLOGY OF *PSEUDOMONAS AERUGINOSA* ANALYSED BY MEANS OF QUANTITATIVE IMMUNOELECTROPHORETIC METHODS

IV Production of Polyvalent Pools of Rabbit Antiserum Against P aeruginosa *(Reference Standard Antibody)*

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Høiby N The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. IV Production of polyvalent pools of rabbit antiserum against *P aeruginosa* (Reference Standard Antibody) Acta path. microbiol. scand. Sect. C, 84 372–382, 1976

The conformity of the immunoprecipitate pattern obtained with a polyvalent *Pseudomonas aeruginosa* antigen (St Ag) and different pools of a polyvalent, purified (IgG and IgA) rabbit antiserum (St Ab) against St Ag has been studied. Ten rabbits were immunized and bled every fifth week. Eight pools of St Ab were produced during nearly four years. All the St Ab pools were mutually compared by means of quantitative immuno-electrophoretic methods. All St Ab pools were found to present St Ag/St Ab immunoprecipitate patterns which qualitatively and quantitatively were similar None of the obtained immunoprecipitates between St Ag and the pooled antisera disappeared during the procedures of purification of IgG and IgA. Quantitative and qualitative comparison of the polyvalent St Ab pools ("titre) was made by counting the total number of immunoprecipitates obtained by St Ag, followed by their identification, and by calculating the mean area of 12 selected and representative immunoprecipitate The abilities of the rabbits to produce antibodies were found to vary individually None of the rabbits produced precipitating antibodies against all the 64 known *P aeruginosa* antigens in St Ag On the average, each of the rabbits lacked precipitating antibodies against 5 of the antigens. By pooling antisera from 3 rabbits out of 8 only 14 per cent of the possible combinations would contain detectable precipitins against all the known antigens of St Ag

Key words *P aeruginosa* immunoelectrophoresis immunization antisera

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Recent developments as regards the quantitative immunoelectrophoretic methods have allowed quantitative studies of complex crude antigen extracts of micro-organisms without preceding purification of individual antigens

(2, 4–7 10–18, 20–21 23–25) The prerequisites of such polyvalent approaches are that complex standard-antigens and corresponding complex standard-antisera are available to be used as reference systems (2) The reproducibility of the immunoprecipitate pattern of such complex reference systems is of importance, notably if these methods are to be used for longitudinal studies (2, 12–17)

Quantitative immunoelectrophoretic methods have shown that *Pseudomonas aeruginosa* has a complex antigenic structure and 64 different antigens in this species have been found by means of these methods (12–17) The polyvalent *P aeruginosa* standard-antigen (St Ag) used to study the serology of these bacteria has been shown to be reproducible, qualitatively (number and identity of antigens) as well as quantitatively (concentrations of the antigens) (17) In this article, the reproducibility of the production of the corresponding polyvalent rabbit standard-antiserum against *P aeruginosa* (St Ab) has been analysed in order to answer the following questions *i* is the St Ag/St Ab immunoprecipitate pattern reproducible if different pools of St Ab are used? *ii* are precipitins against some of the antigens of St Ag lost during the procedures of purification of IgG and IgA from rabbit antisera according to the methods of *Harboe & Ingild* (1973)? *iii* are 1–3 rabbits sufficient for the production of a polyvalent St Ab pool?

MATERIALS AND METHODS

The P aeruginosa Standard Antigen (St-Ag)

St-Ag consists of water-soluble constituents obtained by sonication from 4 different O groups of *P aeruginosa* (12) Seventeen batches of St-Ag have been produced (17) and, in the present work, St-Ag batch 1 and batch 2 are used. These batches have been compared by means of crossed-line immunoelectrophoresis (17) but no qualitative differences have been revealed. Colloid concentrations were 11.8 g/l and 12.1 g/l, respectively. These concentrations were measured by refractometry using human IgG as standard (12, 14) double determinations employing the biuret method (12) and refractometry (14) on 9 different batches of *P aeruginosa* antigens from 9 different O groups (15) has shown a positive correlation (R = 0.8583 $p<0.01$) between the results obtained by these 2 methods. However the results obtained by refractometry were, on an average, 29 per cent higher as this method also measures non-protein antigens.

TABLE 1 *Characterization of 8 Pools of a Concentrated and Purified Antiserum (St Ab) against P aeruginosa Obtained by Repeated Immunization and Bleeding of Originally 10 Rabbits with a Polyvalent P aeruginosa Antigen (St Ag)*

	Batch of St-Ag used for immunization	Number of pooled bleedings (number of rabbits)	Volume of purified St-Ab pools (ml)	Protein concentration of purified St-Ab pools (g/l)	Mean of areas of 12 immunoprecipitates (cm^2 magnification ×9) (see Table 3)*
St Ab pool 1	1	1 (10)	110	29 7	20 5
St Ab pool 2–6	1	7 (10)	525	25 1	23.8
St Ab pool 7 17	1	15 (9)	1475	18.7	26 5
St-Ab pool 18	1	3 (9)	200	29 4	23.5
St Ab pool 19	2	3 (9)	260	27.3	23.3
St-Ab pool 20		7 (9)	575	36.9	15.2
St Ab pool 21	2	1 (8)	89	20.3	22 0
St Ab pool 22	2	1 (8)	47	33 7	18 7

The mean area of the 12 representative immunoprecipitates measured on crossed immunoelectrophoretic plates of St Ag batch against each of the 8 St-Ab pools can be considered an expression of the average titre of the pool the area enclosed by an immunoprecipitate is inversely proportional with the concentration of the antibody (Fig 4 (18 19)) i.e. the smallest mean area corresponds to the highest average titre

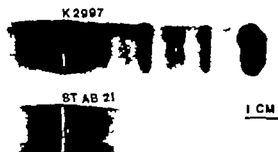


Fig 1 Agarose gel electrophoresis of 10 µl antiserum from rabbit K2997 and of 10 µl St Ab pool 21 (purified and concentrated IgG and IgA see *Materials and Methods*) Technical anode to the right Staining Coomassie brilliant blue

The P aeruginosa *Standard Antibody (St Ab)*

St Ab is a pooled concentrated rabbit antiserum against St Ag (12) It has been produced as previously described (12) by repeted immunization and bleeding according to *Harboe & Ingild* (1973) originally 10 rabbits were included, 8 of which are still alive (Table 1) From January 1972 to September 1975 antisera from each bleeding, occasionally from several bleedings, from the rabbits have been pooled and used for the production of 8 pools of St Ab (Table 1) Before the pooling of antisera from the bleedings used for production of St Ab pool 21 and pool 22 aliquots of antiserum obtained at each of these 2 bleedings were taken from each of the rabbits.

Each pool of St Ab has been purified and concentrated according to *Harboe & Ingild* (1973) and stored at 4 C with 15 mM NaN_3 added (12) By this procedure IgG and IgA are recovered almost quantitatively whereas IgM is lost (9) The purification procedure was controlled by agarose gel electrophoresis (Fig 1) and the protein concentration was measured by refractometry (12 14) (Table 1)

Immunoelectrophoretic Methods

Crossed immunoelectrophoresis of 2 µl St Ag batch 1 or batch 2 against each of the pools of St-Ab or antiserum from each of the 8 rabbits (25 µl/cm²) was run on 5 × 5 cm glass plates (13)

Crossed immunoelectrophoresis with intermediate gel was run on 5 × 5 cm glass plates (14) one µl or 2 µl St Ag batch 1 or batch was run in the 1st dimension electrophoresis. The intermediate gel contained saline as control or St Ab or rabbit antiserum, or various combinations of antisera from 2 or 3 rabbits (40 µl/cm²) The reference gel contained St-Ab (each of the pools) or St Ab pool 21 or pool 22 *before* purification (20 µl/cm)

Immunoplates which were compared quantitatively or qualitatively were always run simultaneously

The areas included by the immunoprecipitates on some of the Immunoplates were measured by planimetry (after magnification × 9) (17) Twelve representative immunoprecipitates were measured on each of these plates. These 12 precipitates represent antigens of various electrophoretic mobility and various sizes of areas enclosed by the precipitates (17) (Table 3 and Fig 3) The relationship between area and antigen/antibody ratio has been shown to be linear or nearly linear (18, 19) The analytical variation (plate-to-plate variation on the same day) expressed as the relative standard deviation is on an average, 6 per cent (range 4 per cent–9 per cent) (17)

Statistical Methods

The F test, Student's t test and Spearman's correlation coefficient R were done using the programmes of a Compucorp 342 Statistician (8, 22) Level of significance 5 per cent (double-tailed tests)

RESULTS

Table 2 shows the results of the qualitative comparison of the 8 pools of St Ab Examples are shown in Fig 2 All pools were mutually compared all electrophoreses were run twice giving identical results, the first time using St Ag batch 1 the second time St Ag batch

TABLE 2 *Occurrence of Precipitating Antibodies against* P aeruginosa *Antigens (St Ag) in 8 Pools of a Polyvalent Rabbit Standard Antibody (St-Ab) against St Ag*

	Precipitate number in the St-Ag/St AB reference system		
	1–53 (62 precipitates)*	1A	1B
St Ab pool 1	+	0	0
St Ab pool 2 6	+	0	0
St Ab pool 7–17	+	0	0
St Ab pool 18		0	+
St Ab pool 19	+	0	+
St Ab pool 20	+	+	+
St Ab pool 21	+	+	0
St-Ab pool 22	+	+	0

The enumeration of the St Ag (batch 1)/St-Ab (pool 2–6) reference system has been published previously (12)

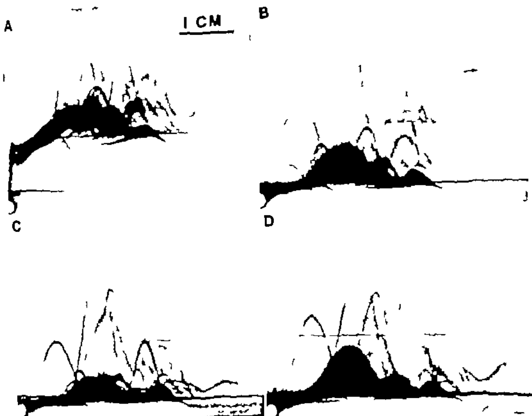


Fig. 2. Crossed immunoelectrophoresis with intermediate gel of St Ag batch 2 against St Ab pool 21 in the reference gel. In the intermediate gel *A*: saline as control, *B*: St-Ab pool 1, *C*: St-Ab pool 18, and *D*: St-Ab pool 22. Nearly all the antigens of St Ag have been precipitated by each of the St Ab pools in the intermediate gels (*B-C-D*) illustrated by absence of some of the precipitates in the reference gels and reactions of identity between the remaining precipitates in the reference gels and the precipitates in the intermediate gels. The only exception was the very weak precipitate number 1 A which is only visible in the reference gels on photography 2 B (arrow) and 2 D; this precipitate could not be revealed in the intermediate gels of Fig. 2 B and 2 C. Technical: 1. dimension electrophoresis: anode to the right; 2. dimension electrophoresis: anode at the top. Staining: Coomassie brilliant blue.

2. The vast majority of precipitating antibodies against St Ag are seen to be present in all pools of St Ab. Two precipitins (1 A and 1 B, Fig. 3) were not detectable in all pools (Table 2). If however St Ab pool 7–17 were concentrated to 1/5 volume (Amicon Concentrator B-15, Amicon, Holland) these precipitins could be traced.

The immunoprecipitate patterns obtained by St-Ag batch 2 against the 8 different pools of St Ab (8 immunoplates) were compared quantitatively with the immunoprecipitate patterns obtained at 8 repeated determinations of St Ag batch 2 against one pool of St Ab (8 immunoplates). A selection of 12 representative immunoprecipitates (Fig. 3) were measured on each of these 2 × 8 plates and the results were compared by means of the F-test and the t test. No significant differences were found (Table 3). There was a significant negative correlation between the mean areas of the 12 precipitates (Table 1) per St-Ab pool and the protein concentrations of the pools ($R = -0.8335$, $p = 0.02$).

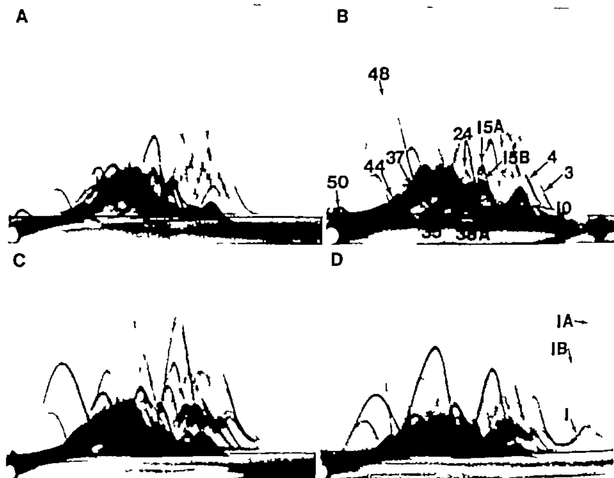


Fig 3 Crossed immunoelectrophoresis of St Ag batch 2 against *A* St Ab pool 1 *B* St-Ab pool 2–6, *C* St Ab pool 19 and *D* St Ab pool 20 Twelve immunoprecipitates are indicated by arrows in *B* and 3 in *D* (see text) Technical as in Fig 2.

When St Ag batch 2 was run against various concentrations of St Ab pool 7–17 the relationship between the mean area of the 12 immunoprecipitates and the concentration of St Ab followed a slightly bent line (Fig 4) The curve fits the relation X × Y = 300 *i.e* X and Y are inversely proportional.

Identification of the precipitates in all the St Ab pools was secured by mixing the pools in series, as shown in Fig 5

Two pools of St Ab (pool 21 and 22) were compared with the pooled antisera from the corresponding bleedings *before* the purification and concentration procedures in order to reveal whether these procedures were accompanied by disappearance of precipitins against some of the antigens of St Ag *i.e* qualitative changes of the immunoprecipitate pattern (Fig 6) These comparisons were done twice using 1 μl and 2 μl St Ag batch 2 similarly the pooled antisera from these bleedings and the St Ab pool 21 and 22 were compared with St Ab pool 20 No qualitative differences between the pooled antisera and the corresponding St Ab pools *after* purification could be revealed

Two separate antisera from each of the 8 rabbits were taken from bleedings numbers 37 and 38 respectively before pooling and purification These antisera were analysed in the St Ag batch 1/St Ab pool 7–17 reference system and also in the St Ag batch 2/St Ab pool 20 reference system (Fig 7) For comparison 1 μl and 2 μl St Ag were used and each comparison was done twice.

TABLE 3 *Area (cm² Magnification × 9) of 12 Precipitates in the St Ag/St Ab Reference Pattern. The Results from 8 Crossed Immunoelectrophoreses of St-Ag Batch 2 against 8 Different Pools of St Ab (Pool 1 2-6 7 17 18 19 20 21 and 22) are Compared with the Results from 8 Repeated Crossed Immunoelectrophoreses of St-Ag Batch 2 against One Pool of St Ab (Pool 7–17)*

		Precipitate number in the St-Ag/St-Ab reference system											
		3	4	10	15A	15B	24	35	37	38A	44	48	50
Ab eight different pools	area*												
	mean	18.5	10.2	13.5	21.0	13 1	32.5	15.3	54.0	8.2	11 4	76.9	2.4
	SD	3.8	4 1	5.4	7.0	4.5	5.7	4.8	16 3	1.5	2.3	24 8	1.6
	% (rel SD)	21%	40%	40%	33%	34%	18%	31%	30%	18%	20%	32%	67%
Ab pool 7-17 × 8	area												
	mean	19.5	13.8	18.2	25.9	15.0	31.9	15.0	65.0	7 1	10 4	87 1	2.7
	SD	4.3	3.6	3.9	5 6	3.2	5 4	5 6	16.7	2.2	2.6	19.2	1.3
	% (rel. SD)	22%	26%	21%	22%	21%	17%	37%	26%	31%	25%	22%	48%
t-test		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
F-test		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n	n.s.

rel SD relative standard deviation. Level of significance 5 per cent. n.s. not significant.

* As compared with similar previously published results (12) the areas are smaller due to higher concentrations of St-Ab in the gel and another St Ag batch. Precipitate number 38A has been measured instead of number 14 the latter being difficult to measure in some of the St-Ab pools.

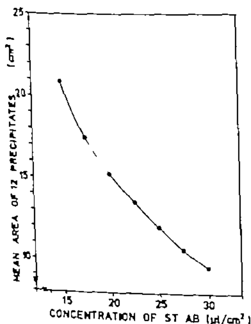


Fig 4 Mean area (magnification × 9) of 12 immunoprecipitates (Table 3) plotted against concentration of St-Ab in the gel. Results of crossed immunoelectrophoreses of 2 µl St-Ag batch 2 against various concentrations of St-Ab pool 7 17 in the second dimension gel.

Additionally crossed immunoelectrophoreses were run with 2 µl St Ag batch 1 as well as with 2 µl St Ag batch 2 against antiserum from each rabbit in the second dimension gel. None of the rabbits had detectable precipitins against all the known *P aeruginosa* antigens in St Ag and 12 (19 per cent) of the reference precipitins were not present in all of the rabbit antisera (Table 4) In all cases, antisera from the 2 different bleedings from the same rabbit showed the same immunoprecipitate pattern.

The 28 possible different combinations of 2 rabbit antisera out of 8 and the 56 possible different combinations of 3 rabbit antisera out of 8 were analysed in the St-Ag batch 2/St-Ab pool 20 reference system. In accordance with the results shown in Table 4 it was found that 9 (14 per cent) of the reference precipitins were not present in all the 28 combinations of 2 antisera. Each of these 28 combinations lacked, on an average, 2.5 precipitins (range 1–5 precipitins)

In accordance with the results shown in Table 4 it was found that 7 (11 per cent) of the reference precipitins were not present in

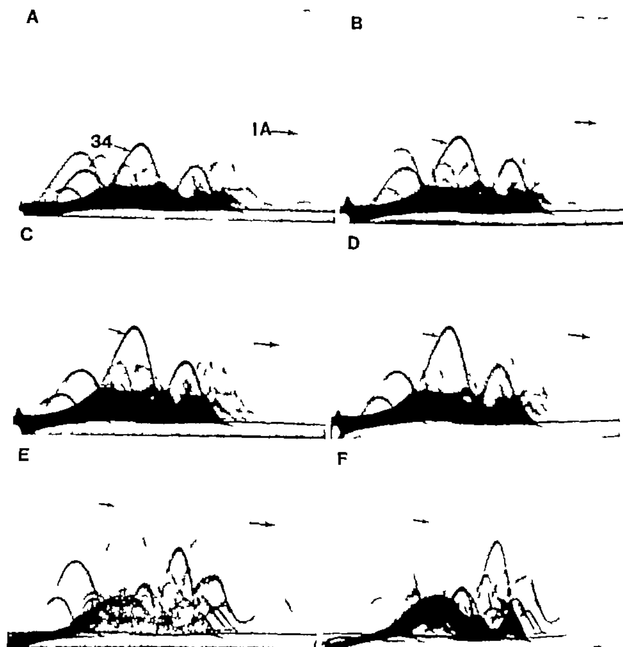


Fig 5 Crossed immunoelectrophoresis of St Ag batch ? against a series of mixtures of St-Ab pool 20/St Ab pool 7–17 (µl per cm²) *A* 25/0 *B* 20/5 *C* 15/10 *D* 10/15 *E* 5/20, *F* 0/25 Two precipitates are indicated by arrows, one of these (1 A) is not visible in *F* the other can be seen in all 6 plates. Technical as in Fig 2

all the 56 combinations of 3 antisera. Each of these 56 combinations lacked, on an average 1 4 precipitins (2 per cent, range 0–3 precipitins) and only 8 of the combinations (14 per cent) contained all 64 precipitins.

DISCUSSION

According to the results obtained in the present study the methods by which antisera can be produced and IgG and IgA be isolated according to *Harboe & Ingild* (1973) can be used for the production of large pools of the polyvalent St Ab and give reproducible results. Although the reference St Ag/St Ab precipitate pattern of crossed immunoelectrophoresis is complex, it remained stable throughout the 8 pools, i.e for nearly 4 years of repeated immunization and bleeding of 10

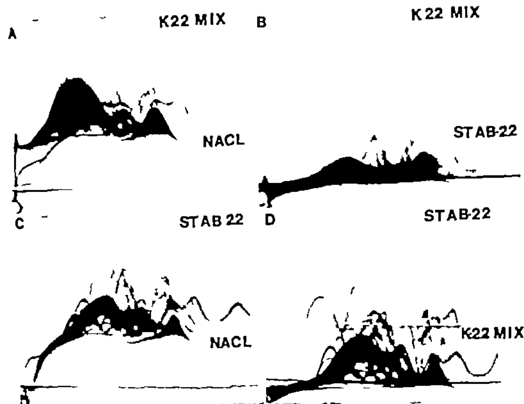


Fig 6 Crossed immunoelectrophoresis with intermediate gel of St-Ag batch 2 against the pooled antisera of the 8 rabbits K2991 K2998 *before* the purification procedures in the reference gel of *A* and *B* and in the intermediate gel of *D* (K 22 MIX) and *after* the purification procedures (St-Ab-22) in the intermediate gel of *B* and in the reference gel of *C* and *D* The intermediate gel of *A* and *C* contained saline as control (NACL) All the antigens of St-Ag have been precipitated in the intermediate gels of *B* and *D* illustrated by absence of some of the precipitates in the reference gels of *B* and *D* compared with the controls (*A* and *C*) and reactions of identity between the remaining precipitates in the reference gels and the precipitates of the intermediate gels of *B* and *D* Technical as in Fig. 2

rabbits, two of which succumbed though any notable subsequent change in the reference precipitate pattern did not occur

The results recorded in Table 4 and Fig 7 show in accordance with results obtained by other authors (1 3 9) that abilities of the rabbits to produce antibodies vary individually whereas any notable differences between antisera from the two successive bleedings from each rabbit were not found. In accordance with other authors (1 2) it appears from these results that it is advisable to immunize several rabbits to obtain a polyvalent standard antibody pool. Even if 3 rabbits were immunized and bled according to the present methods, it cannot be precluded that precipitins against some of the antigens may be lacking.

The fact that different pools of St Ab were used did not give rise to major problems. Although the protein concentrations of the pools differed, the identity of precipitins in the different pools could be established by means of crossed immunoelectrophoresis with intermediate gel (Fig 2) and by mixing the pools in series of crossed immunoelectrophoreses (Fig 5) (3) By means of these methods, antibody pools produced from differ

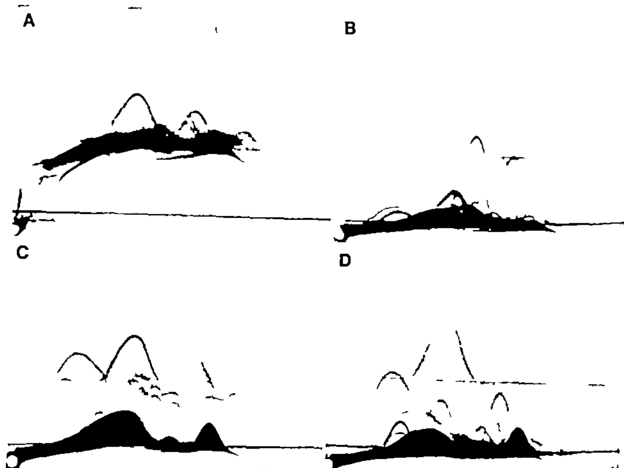


Fig 7 Crossed immunoelectrophoresis with intermediate gel of St Ag batch 2 against St-Ab pool 20 in the reference gel and in the intermediate gels *A* saline as control, *B* antiserum from rabbit K2991 *C* antiserum from rabbit K2994 and *D* antiserum from rabbit K2997 Most of the antigens of St Ag are precipitated by antibodies in the intermediate gels of *B* *C* and *D* Some of the precipitates of the reference gels are not influenced by the antibodies in the intermediate gels reflecting absence of antibodies in the intermediate gels against the corresponding antigens of St Ag (see Table 4) Technical as in Fig 2

rabbits in different laboratories can be compared and thus, standardized nomenclatures of the precipitates can be established (2 3)

The correlation between the mean area of precipitates and the St Ab concentration and between the mean area of precipitates and the protein concentration of the St Ab pool indicate that the mean area of a selection of representative precipitates could be a useful expression of antibody titre of polyvalent antibody pools. Thus, the most informative characteristics of St Ab pools are probably the total number and identity of precipitates with a given St Ag batch and the mean area of a selection of representative precipitates under specified experimental conditions.

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REFERENCES

1 *Axelsen N H & Svendsen P J* Candida precipitins characterized by a modified antigen antibody crossed electrophoresis. In *Peeters H* (Ed) Protides of the Biological Fluids. Proceedings of the 19th Colloquim, 1971 Pergamon Press Oxford & New York 1972 p 561-564

2 *Axelsen N H* Quantitative immunoelectrophoretic methods as tools for a polyvalent approach to standardization in the immuno-

TABLE 4 *Occurrence of Precipitating Antibodies against P. aeruginosa Antigens (St-Ag) in Antisera from each of 8 Rabbits Used for Production of the Pooled Polyvalent Rabbit Standard Antibody (St Ab) against St-Ag*

Precipitate number in the St-Ag/St-Ab reference system	Rabbit								Number of antisera containing the precipitins
	K2991	K2992	K2993	K2994	K2995	K2996	K2997	K2998	
1A	0	0	0	0	0	0	+	0	1
1B	+	+	0	+	0	+	+	+	6
5	+	0	+	+	+	+	+	+	7
8A	0	0	0	0	0	+	+	+	3
18A	0	+	+	+	+	0	0	0	4
33	+	+	+	0	0	0	0	0	3
34	+	0	0	0	+	0	0	+	3
39	0	+	0	0	+	0	+	+	4
43	0	+	+	0	0	0	+	+	4
48	+	+	0	0	+	+	+	+	6
49	+	+	+	0	+	+	+	+	7
50	+	+	+	0	+	+	+	+	7
Remaining 32 precipitates	+	+	+	+	+	+	+	+	8
Number of non-visible precipitates	5	4	6	9	5	6	3	3	

chemistry of *Candida albicans*. Infect. and Immun. 7 949–960, 1973

3. *Axelsen, N. H. Bock, E. & Kroll, J.* Comparison of antibera. Scand. J. Immunol. 2 Suppl. 1 161–164 1973.

4. *Axelsen N. H. Harboe M., Closs O. & Godal, T.* BCG antibody profiles in tuberculoid and lepromatous leprosy. Infect. and Immun. 9 952 958 1974

5. *Caldwell, H. D. Kuo C.-C. & Kenny G. E.* Antigenic analysis of Chlamydia by two-dimensional immunoelectrophoresis. I Antigenic heterogeneity between *C. trachomatis* and *C. psittaci*. J. Immunol. 115 963–968, 1975

6. *Caldwell, H. D. Kuo C.-C. & Kenny G. E.* Antigenic analysis of Chlamydia by two-dimensional immunoelectrophoresis. II. A trachoma-LGV-specific antigen. J. Immunol. 115 969–975 1975

7. *Closs, O. Harboe M. & Wassum A. M.* Cross-reactions between Mycobacteria. I Crossed immunoelectrophoresis of soluble antigens of *Mycobacterium lepraemurium* and comparison with BCG. Scand. J. Immunol. 4 Suppl. 2, 173 183, 1975

8. *Diem K.* (Ed.) Documenta Geigy Scientific Tables. J. R. Geigy S. A. Basle 1962 pp. 32 35 & 40-41 & 66

9. *Harboe N. & Ingild A.* Immunization, isolation of immunoglobulins, estimation of antibody titre. Scand. J. Immunol. 2 Suppl. 1 161 164, 1973

10. *Holmberg, K. Nord C.-E. & Wadström T.* Serological studies of *Actinomyces israelii* by crossed immunoelectrophoresis standard antigen-antibody system for *A. israelii*. Infect. and Immun. 12 387–397 1975.

11. *Holmberg, K., Nord C. E. & Wadström T.* Serological studies of *Actinomyces israelii* by crossed immunoelectrophoresis taxonomic and diagnostic applications. Infect. and Immun. 12 398–403 1975.

12. *Høiby N. & Axelsen N. H.*, Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol. scand. Sect. B 81 298–308 1973

13. *Høiby N.* *Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta path. microbiol. scand. Sect. B, 82 551–558, 1974

14. *Høiby N.* Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. Scand. J. Immunol. 4 Suppl. 2, 187–196, 1975

15. *Høiby N.* The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. I. Comparison of thirteen O groups of *Ps. aeruginosa* with polyvalent *Ps. aeruginosa* antigen-antibody reference system. Acta path. microbiol. scand. Sect. B 83 321 327 1975.

Høiby N The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. II Comparison of the antibody response in man against thirteen O groups of *Ps aeruginosa*. Acta path. microbiol scand. Sect. B *83* 328–334 1975

Høiby N The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. III Reproducibility of a polyvalent *P aeruginosa* reference Standard Antigen Acta path. microbiol scand. Sect B *83* 433–442 1975

Martin G R., Warocquier R & Boulanger P A Quantitation of Adenovirus soluble antigens by crossed immunoelectrophoresis application to serological characterization of mutants. Intervirol *5* 162–172 1975

Mrachin Clarke H G & Freeman T A quantitative immuno-electrophoresis method (Laurell electrophoresis) *In Peeters H* (Ed.) Protides of the Biological Fluids, vol. 14 Elsevier Amsterdam 1967 p. 503–509

Owen P & Salton M R J Antigenic and enzymatic architecture of *Micrococcus lysodeikticus* membranes established by crossed immunoelectrophoresis. Proc. Nat. Acad. Sci U.S.A. *72* 3711–3715 1975

21 *Roberts D B Wright G L Jr Affronti, L F & Reich M* Characterization and comparison of Mycobacterial antigens by two-dimensional immunoelectrophoresis. Infect. and Immun. *6* 564–573 1972.

22 *Therkelsen A J Medicinsk Statistik* Akademisk Boghandel, Århus 1968, pp 69–71 79 & 102–110

23 *Thirkill C E. & Kenny G E.* Serological comparison of five arginine-utilizing Mycoplasma species by two-dimensional immunoelectrophoresis. Infect. and Immun. *10* 624–632 1974

24 *Thirkill C E. & Kenny G E.* Antigenic analysis of three strains of *Mycoplasma arginini* by two-dimensional immunoelectrophoresis. J Immunol. *114* 1107–1111 1975

25 *Vestergaard B F* Crossed immunoelectrophoretic characterization of herpesvirus hominis type 1 and 2 antigens. Acta path. microbiol. scand. Sect. B, *81* 808–810 1973

Acta path. microbiol. scand. Sect. C, 84: 383–389, 1976

THE SEROLOGY OF *PSEUDOMONAS AERUGINOSA* ANALYSED BY MEANS OF QUANTITATIVE IMMUNOELECTROPHORETIC METHODS

V. *Thermostability, Resistance to Degradation by Plasmin Activity and Storage Conditions of a Polyvalent* P. aeruginosa *Reference Standard Antigen*

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Høiby, N. The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. V. Thermostability, resistance to degradation by plasmin activity and storage conditions of a polyvalent *P. aeruginosa* reference standard-antigen. Acta path. microbiol. scand. Sect. C, 84: 383–389, 1976.

The stability of a polyvalent *Pseudomonas aeruginosa* antigen (St-Ag) was analysed under different experimental conditions by quantitative immunoelectrophoretic methods. St-Ag did not undergo proteolytic degradation by plasmin which is present in the corresponding polyvalent rabbit antiserum (St-Ab) against St-Ag. Storage at −80° C for up to 4 years caused no qualitative changes in St-Ag and, after 1 year, only minor quantitative changes could be revealed. Repeated thawing and freezing caused no changes in St-Ag whereas storage at 4° C or at room temperature caused gradual degradation of most of the antigens of St-Ag, beginning after 1 week. Heating up to 40° C for 2 min did not change St-Ag, but heating for longer periods of time or at higher temperatures gradually destroyed most of the antigens of St-Ag. The most thermostable antigen in St-Ag was number 37 which is related to the group specific O antigens.

Key words: *P. aeruginosa*; immunoelectrophoresis; thermolability; antisera; plasmin.

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The application of quantitative immunoelectrophoretic methods in the serology of *Pseudomonas aeruginosa* has revealed a complex antigenic structure of this species (6–12). The polyvalent reference *P. aeruginosa* Standard-Antigen/Standard-Antibody (St-Ag/St-Ab) system used in these studies has been found to give reproducible results (11, 12). The St-Ab was shown to be stable during storage for nearly 4 years (5, 12).

Quantitative immunoelectrophoretic methods can be used in the analysis of degradation of proteins since model studies have revealed

characteristic changes in immunoprecipitate morphology during degradation (1 2) In the present work quantitative immunoelectrophoretic methods have been used to investigate the stability of the *P aeruginosa* reference St Ag *i* under different storage conditions, *ii* during handling procedures in the laboratory *iii* resistance of St Ag to proteolytic activity caused by the presence of plasmin in rabbit immunoglobulin preparations purified by the methods of *Harboe & Ingild* (1973) which have been used to produce St Ab (3 6)

MATERIALS AND METHODS

The St Ag/St Ab Reference System

This reference system has been described previously (6 11 12) St Ag consists of water-soluble constituents obtained by sonication of 4 strains of *P aeruginosa* (6 11) St Ag batches 1 2 12 15 and batch 3–15 (13 pooled batches) were used in the present work colloid concentrations (refractometry using human IgG as standard) 11.8 g/l 12.1 g/l 13 4 g/l 14 4 g/l and 11.8 g/l (6, 11 12) No qualitative differences between these batches have been found (11) (qualitative number and identity of antigens, quantitative concentration of the antigens) St Ab pool 7–17 was employed throughout the study (12)

Immunoelectrophoretic Methods

Crossed immunoelectrophoresis and *crossed-line immunoelectrophoresis* (absorption of antibodies *in situ*) of 2 µl of St Ag against St Ab in the second dimension reference gel (25 µl/cm²) was run on 5 × 5 cm glass plates (7 8) The intermediate gel in crossed line immunoelectrophoresis contained St Ag (20 µl/cm²) or saline as control. The areas of immunoprecipitates which are proportional with the antigen/antibody ratio were measured by planimetry after magnification × 9 (11 12) The analytical variation (relative standard deviation) on the same day (plate-to-plate variation) is, on the average 6 per cent (range 4 9 per cent) and the day to-day variation is, on the average, 15 per cent (range 10–19 per cent) (11)

Proteolytic Activity in St Ab Caused by Plasmin (3)

was measured in 9 cm petri dishes containing 6.2 ml fibrin clots (0 1 per cent bovine fibrinogen mixed with bovine thrombin (20 NIH/ml) in Tris-HCl 0 05 M NaCl 0 10 M, final pH 7 4 kindly donated by dr *Inge Clemmesen* Aliquots of 10 µl of St Ab or saline as control were incubated on the fibrin clot at 35 C and at 12 C for 17 h. Plasmin activity was recorded qualitatively as dissolution of the fibrin clot Twofold dilutions of aprotinin (polyvalent protease inhibitor Trasylol® 10,000 U/ml Bayer Leverkusen, West Germany) were added to St Ab to neutralize the proteolytic activity of plasmin (3)

Storage Conditions and Examination of Heat Stability of St Ag

St Ag batches 1 2 12 part of batch 15 and batch 3–15 were stored at –30 C in 100 µl aliquots in small sealed plastic tubes or in 1 ml aliquots in sealed glass tubes. Aliquots of St Ag batch 15 were stored at 4 C and at room temperature (21 C) 500 µl St-Ag batch 15 was lyophilized and stored at 21 C. Other aliquots of St-Ag batch 15 were subject to *i* daily repeated thawing (4 C) and freezing (–30 C) *ii* heating on waterbath at 40 C for 2 min, *iii* at 40° C for 30 min *iv* at 50 C for 2 min, *v* at 60 C for 30 min, *vi* at 100 C for 1 h, *vii* autoclaving at 120 C for 1 h, and *viii* at 120 C for 2 h. Two µl aliquots of St Ag batch 15 treated as described above were run in crossed immunoelectrophoresis against St Ab simultaneously 2 µl untreated St-Ag batch 15 was run as control.

Ten identical immunoplates of 2 µl St Ag batch 12 against St Ab were run after 14 days of storage at –30 C and again after storage of the batch for 14 months at –30 C. The areas of twelve immunoprecipitates on each of these 2 × 10 immunoplates were measured (Fig 3 A) and compared by means of the t test for paired comparisons using a Compucorp 342 Statistician (4 13)

RESULTS

Incubation of St Ab on a fibrin clot showed plasmin activity at 35° C as well as at 12 C. The plasmin activity could be neutralized by addition of 10 U aprotinin per ml St Ab without any subsequent changes in the St Ag/St Ab immunoprecipitate pattern.

The stability of St Ag during storage at –30 C for 14 months compared to the stability during storage at –30 C for 14 days is seen from the results shown in Table 1 After 14 months at –30 C, the areas of 9 precipitates had diminished and the areas of 3 precipitates were increased on an average the mean areas of the 12 precipitates showed a small (7 per cent) but significant decrease ($p<0.01$) which however did not exceed the analytical day to-day variation The quan-

TABLE 1 The Results from 10 Repeated Crossed Immunoelectrophoreses of St-Ag Batch 12 after 14 Days Storage at −30° C Compared with the Results from 10 Repeated Crossed Immunoelectrophoreses of St-Ag Batch 12 after 14 Months Storage at −30° C

	Area	Number of precipitate in the St-Ag/St-Ab reference system: 7	4	10	14	15A	15B	24	35	37	44	48	50	Mean of the 12 precipitates
St-Ag batch 12 after 14 days at −30° C	mean	37.2	50.8	22.1	26.3	79.2	45.5	56.6	34.0	75.9	29.1	131.8	11.6	48.3
	SD	7.7	5.5	4.6	5.8	12.8	8.4	7.5	6.0	11.9	3.2	5.2	2.0	
(10 immunoplates) Standardized area 14 days (S-area 14 days)	mean	0.77	0.64	0.46	0.54	1.64	0.94	1.17	0.70	1.57	0.60	2.73	0.24	1.00
	SD	0.16	0.11	0.10	0.12	0.27	0.17	0.16	0.12	0.25	0.07	0.11	0.04	
St-Ag batch 12 after 14 months at −30° C	mean	35.5	24.2	22.4	29.1	75.3	38.8	48.9	28.6	69.4	25.9	134.1	8.9	44.9
	SD	5.4	2.8	2.2	5.4	10.1	4.4	8.5	4.0	11.3	3.5	15.4	1.5	
(10 immunoplates) Standardized area 14 months (S-area 14 months)	mean	0.74	0.54	0.50	0.65	1.68	0.86	1.09	0.64	1.58	0.58	2.99	0.20	1.00
	SD	0.08	0.08	0.05	0.12	0.23	0.10	0.18	0.09	0.25	0.08	0.34	0.03	
$\frac{\text{S-area 14 months}}{\text{S-area 14 days}} \times 100\%$		96 %	84 %	109 %	119 %	102 %	91 %	93 %	91 %	100 %	96 %	109 %	83 %	
t-test (S-areas)	p	n.s.	$<.05$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	$<.05$	$<.02$	

SD standard deviation, n.s.: not significant, p probability of significant difference.

The area (cm² magnification × 9) of 12 precipitates in the St-Ag batch 12/St-Ab pool 7–17 reference pattern was measured on each immunoplate. The quantitative proportions between the 12 precipitates after 14 days of storage and after 14 months of storage are standardized by dividing the area of each precipitate with the mean area of all the 12 precipitates of all the 10 corresponding immunoplates (standardized area).

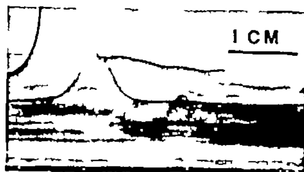


Fig 1 Crossed line immunoelectrophoresis of St Ag batch 3–15 in the well and St Ag batch 1 in the intermediate gel against St Ab pool 7–17 in the reference gel (absorption of antibodies *in situ*) All the precipitates of the reference pattern have been removed or elevated and corresponding straight precipitate lines showing reaction of identity with the elevated reference precipitates are seen in the gel (control with saline in the intermediate gel is not shown) Technical 1st dimension electrophoresis anode to the right 2nd dimension electrophoresis anode at the top Staining Coomassie brilliant blue)

titative proportions betwen the 12 antigens (precipitates) after 14 days of storage and after 14 months of storage at –30 C were calculated, using the mean area of all the 12 precipitates of all the 10 corresponding immunoplates (Table 1) as unit (built in reference) (Standardized area, Table 1) It appears from Table 1 that differences in the quantitative proportions of the antigens were only small after storage at –30 C for 14 months.

The stability of St Ag at –30 C is also illustrated in Fig 1 which shows that St Ag batch 1 after storage at –30 C for 4 years is still able to absorb all *precipitins in* St Ab against St Ag batch 3–15 which has been stored at –30° C for 1 year

Lyophilization of St *Ag batch* 15 *and* rehydration after 1 week did not change the precipitate pattern qualitatively or quantitatively Thawing *and freezing* of St Ag batch 15 repeated seventeen times did not change the precipitate pattern qualitatively or quantitatively

Storage of St Ag batch 15 at 4° C and at room temperature caused no changes in the precipitate pattern after 2 days. After 1 week at room temperature however precipitate number 14 disappeared and, after 1 week at 4° C precipitate number 8A disappeared (Fig 2) During storage for 1 year at 4° C and especially at room temperature most of the precipitates gradually disappeared (Fig 2) one of the few precipitates to remain after 1 year at room temperature was number 37 which was partially degraded as indicated by the morphology (double peak) of *the precipitate (Fig 2 F) No bacterial* contamination occurred during these experiments

The results of the experiments with a view to the thermostability of St Ag batch 15 are shown in Fig 3 Heating at 40 C for 2 min did not change the precipitate pattern, whereas heating for 30 min at 40° C resulted in disappearance of precipitate number 15A and a change in morphology of number 37 (Fig 3 B) Heating up to 50 C for 2 min resulted in disappearance of precipitate number 15A and 15B Heating up to higher temperatures as shown in Fig 1 caused gradual disappearance of most of the precipitates. Only precipitate number 37 did not disappear after 2 h at 120 C although the morphology of this precipitate changed markedly and part of the precipitate disappeared

DISCUSSION

According to *Bjerrum et al* (1975) plasmin is responsible for the proteolytic activity present in immunoglobulin preparations (*i.e* St Ab) which have been purified according to *Harboe & Ingild (1973) Bjerrum et al.* (1975) have shown that plasmin activity can cause partial degradation of some proteins *during the electrophoresis,* resulting in extra precipitation arcs of congruent shape. The present results show that the *P aeruginosa* antigens in St Ag are stable to the proteolytic activity of plasmin in St Ab addition of

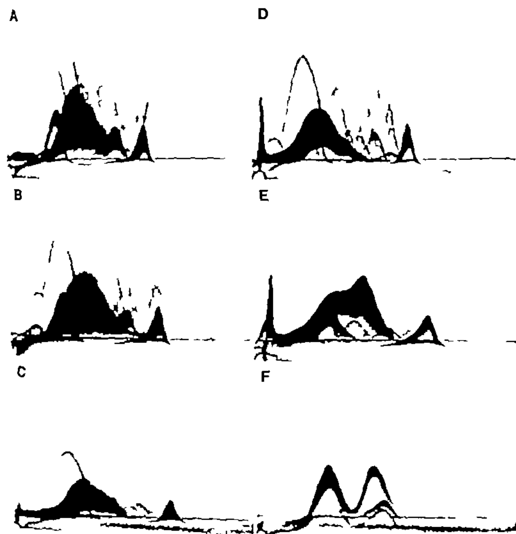


Fig 2 Crossed immunoelectrophoresis of St Ag batch 13 against St Ab pool 7 17 After storage of St-Ag at 4 C, A for 3 months, B for 6 months, and C for 1 year The arrows indicate precipitate number 10 and number 37 After storage of St Ag at room temperature, D for 3 months, E for 6 months and F for 1 year Compare with Fig. 3 A. In Fig 2 F only precipitate number 37 (grossly changed morphology) and 2 unidentified precipitates are visible. Technical as in Fig. 1

protease inhibitors to St Ab is therefore not indicated (3)

It appears that crossed immunoelectrophoresis is a sensitive method by which to detect and follow denaturation or degradation of single microbial antigens in complex mixtures. The changes in immunoprecipitate patterns which occur during degradation of proteins have been studied in model experiments by *Bjerrum & Bøg-Hansen* (1975) the various changes in morphology migration velocity staining intensity and disappearance of precipitates described by these authors are also seen in the St-Ag/St Ab *precipitate* pattern (Fig 2 & 3)

It appears from Figs. 2 & 3 that antigen

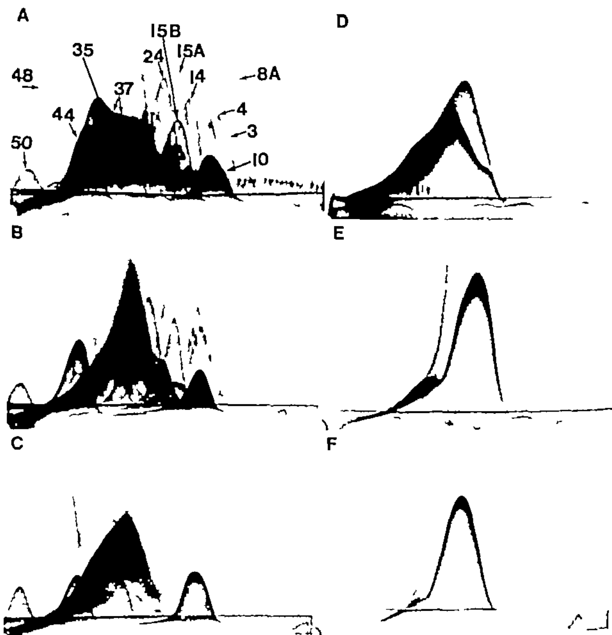


Fig 3 Crossed immunoelectrophoresis of St-Ag batch 15 against St Ab pool 7–17 A control plate, 13 precipitates are indicated by arrows. St Ag heated before electrophoresis, B at 40 C for 30 min, C at 60 C for 30 min, D at 100 C for 1 h E at 120 C for 1 h, F at 120 C for 2 h. Precipitate number 15A has disappeared and 15B has diminished and number 37 has changed morphology in B. In F only precipitate number 37 (grossly changed morphology) is visible Technical as in Fig 1

number 37 is the most heat stable antigen in St Ag The morphology of this precipitate on control plates (Fig 3 A) suggests that it consists of electrophoretically heterogenic molecules (2) Moreover this precipitate is the only precipitate in the St Ag/St Ab reference pattern which is stained by the perodic acid-Schiff reaction (polysacharides) as well as by Coomassie brilliant blue (proteins) whereas neither this precipitate nor any of the other precipitates are stained by Sudan black (lipids) (14) These features indicate that antigen number 37 is a polysaccharide-protein complex and in accordance with previous results, it is probably related to the O antigens of *P aeruginosa* (9)

The present results show that St Ag remain rather stable during normal laboratory handling and storage procedures even at room temperature it remained stable for 2 days. Storage at 4 C or at room temperature for more than 2 days should be avoided, however as a slow degradation of some of the antigens begins after that time especially at room temperature. The reason for the degradation of some of the antigens after 1 week or more at these temperatures is uncertain, although autodigestion due to proteolytic enzymes could be suspected. However this slow degradation should have no influence on the reproducibility of the St-Ag/St Ab precipitate pattern if St Ag is stored and handled in accordance with the present results.

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REFERENCES

1 *Axelsen, N H* Quantitative immunoelectrophoretic methods as tools for a polyvalent approach to standardization in the immunochemistry of *Candida albicans* Infect. and Immun. 7 949–960 1973

2 *Bjerrum O J & Bøg-Hansen, T C* Analysis of partially degraded proteins by quantitative immunoelectrophoresis Scand J Immunol. 4 Suppl 2, 89–99 1975

3. *Bjerrum O J Ramlau J Clemmesen I Ingild, A & Bøg-Hansen T C* An artefact in quantitative immunoelectrophoresis of spectrin caused by proteolytic activity i antibody preparations. Scand. J Immunol 4 Suppl. 2, 81 88, 1975

4 *Diem* A (Ed) Documenta Geigy Scientific Tables. J R Geigy S A Basle 1962, p 32

5 *Harboe N & Ingild A.* Immunization, isolation of immunoglobulins, estimation of antibody titre. Scand. J Immunol. 2 Suppl. 1 161–164 1973

6. *Høiby N & Axelsen, N H.* Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol. scand. Sect. B *81* 298–308 1973.

7 *Høiby N Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta path. microbiol scand. Sect. B, *82* 551–558, 1974

8. *Høiby N* Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. Scand. J Immunol. *4* Suppl. 2, 187 196, 1975

9 *Høiby N* The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. I Comparison of thirteen O groups of *Ps. aeruginosa* with a polyvalent *Ps aeruginosa* antigen-antibody reference system. Acta path microbiol scand. Sect. B, *83* 321–327 1975

10 *Høiby N* The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. II Comparison of the antibody response in man against thirteen O groups of *Ps. aeruginosa* Acta path. microbiol. scand. Sect. B, *83* 328–334 1975

11 *Høiby N* The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. III. Reproducibility of a polyvalent *P aeruginosa* reference Standard-Antigen. Acta path. microbiol. scand Sect. B, *83* 433–442, 1975.

12. *Høiby N* The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. IV Production of polyvalent pools of rabbit antiserum against *P aeruginosa* (reference Standard-Antibody) Acta path. microbiol. scand. Sect. C *84* 372 382, 1976

13 *Thorkelsen A J Medicinsk Statistik* Akademisk Boghandel, Århus 1968 p 82–83

14 *Uriel J* The characterization reactions of the protein constituents following electrophoresis or immunoelectrophoresis in agar *I* Grabar P & Burtin, P (Ed.) Immunoelectrophoretic Analyses Elsevier Amsterdam 1964 p. 30–57

EFFECT OF 2-MERCAPTOETHANOL ON THE MIXED LEUKOCYTE REACTION IN MAN

Probable Effect on Blastogenic Factor Stabilization

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Axelsson, J A., Källén B Nilsson, O & Tropé C. Effect of 2 mercaptoethanol on the mixed leukocyte reaction in man. Probable effect on blastogenic factor stabilization. Acta path. microbiol scand. Sect. C, 84 390–396 1976

When 2 mercaptoethanol (2 ME) is added to human mixed leukocyte cultures at the beginning of the culture period only a very weak effect is seen. When 2 ME is added after a few days culture, a marked enhancement of cellular transformation occurs. 2 ME can also enhance the effect of blastogenic factor (BF) containing media and it is possible that this effect depends on a stabilization or protection of BF preventing the break-down of BF in cellular systems

Key words Mixed leukocyte culture 2-mercaptoethanol blastogenic factor

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2 mercaptoethanol (2 ME) has been shown to enhance various immunological *in vitro* reactions *e g* production of plaque forming cells (3–5) lymphocyte transformation (8) and mixed leukocyte reaction (MLR) (1 9 11 12 16) The mode of action of 2 ME is not clear It has been suggested that it acts by supporting cell survival and viability (*e g* 1) but a more specific effect as a substitute for the function of macrophagic cells has also been suggested (3 4) *Daniele & Rowlands* (6) demonstrated that treatment of lymphocytes at 37 C for 30 minutes with 2 ME resulted in the appearance of receptors for complement in approximately half of the lymphocytes that did not show such receptors before treatment with 2 ME. *Rosenstreich & Wilton* (16) suggested that 2 ME could substitute for one of several of the functions possessed by a soluble macrophage product, the lymphocyte activating factor (LAF) *Melsom et al* (13) demonstrated that another soluble macrophage product, the very labile macrophage cytolytic factor (MCF) could be stabilized by 2 ME.

Most studies of the effect of 2 ME on MLR have used culture systems where optimum conditions were absent, either because of lack of protein in the culture medium (9 11) or lack of adherent cells (1) *Soulillou et al* (17) found an enhancing effect of 2 ME on rat MLR, where proliferation is weak, in the absence of serum or at low serum concentrations *Rode & Gordon* (15) could not replace adherent cells with 2 ME in MLR between human leukocytes.

We here report an effect of 2 ME on human MLR which differs somewhat from that described by earlier authors. When 2 ME was

added at the beginning of the cultures, it produced only a weak effect when it was added after a few days culture, the effect was strong, although variable This is probably due to a protection or activation of a soluble product, a blastogenic factor

MATERIALS AND METHODS

For details of the MLR technique see (10) Leukocytes were separated by dextran from heparinized blood taken from healthy subjects. One-way MLR was obtained by pretreatment of leukocytes from one donor in a pair (stimulator) with mitomycin C. Each culture tube contained 1.5 ml of medium—30 per cent heat-inactivated normal human serum in Parker 199 with heparin and antibiotics added—and 3 × 10⁶ cells Half of the cells were stimulator cells, half were non-mitomycin C treated responder cells. Triplicate tubes were used. The cells were cultured for 7 days at 37 C.

In some experiments, the supernatant from MLR cultures was used as source of blastogenic factor For such cultures, 100 ml Erlenmeyer flasks were often used. Cells were removed by centrifugation at 200 G for 5 minutes followed by second centrifugation at 1000 G for 10 minutes. The medium was usually used fresh, but sometimes it was stored at −80 C without apparent loss of its blastogenic activity The blastogenic effect of the medium was tested on cells from one individual combined with mitomycin treated cells from the same individual (mitA + A) These cultures were run for 2-4 days the medium was then changed to the medium to be tested for blastogenic factor activity and the cultures were continued until day 7

2 ME (Serva, Heidelberg) was diluted with saline to 1 1000 stock solution kept at +4 C up to 4 weeks. Just before use the stock solution was diluted with saline (1 20) and 50 μl of that solution was added to 1.5 ml of culture medium, giving final concentration of 2 7 × 10 M.

DNA synthesis was evaluated as described by Hedberg et al. (10) After one hour' incubation with tritiated thymidine (methyl-³H-thymidine Schwarz/Mann, 1 9 Ci/mM, final concentration 10 μCi/ml) nucleosides and nucleotides were extracted with cold trichloroacetic acid and the residue was dissolved in Soluene 350 (Packard Ltd) and mixed with scintillation fluid. Radioactivity was assayed by 10 minutes counting in Packard Tri-Carb liquid scintillator with external standardization. In order to obtain near-normal distribution of the values (/ 10) cpm were expressed in $\log_{10}$ form. Stimulation in a culture was thus evaluated as the difference between log cpm in "positive" (mitA + B) and in control" (mitB + B) the effect of addition of blastogenic factor containing media or of 2 ME was evaluated in a similar way as $\log_{10}$ differences between experimental group and corresponding control group.

RESULTS

All MLR cultures studied gave strong thymidine incorporation at day 7—stimulation index (compared with non-mixed cells) was usually around ten, but some were weaker (minimum four) and some were stronger (maximum twenty) Addition of 2 ME at the beginning of the MLR cultures results, on the average, in a very slight stimulation of thymidine incorporation at day 7 Preliminary studies using 18 combinations of cells showed that the mean log difference between the thymidine incorporation in MLRs treated with 2 ME and identical cultures without 2 ME was 0 15 ± 0 02, representing approximately 40 per cent increase in registered counts.

Subsequently we studied the effect of 2 ME when added at different times during the culture period. 2 ME was left in the MLRs until the end of the culture period at day 7 Fig 1 gives the individual results of ten such experiments and a graph showing the mean effect at different days of the addition of 2 ME. Thymidine incorporation at day 7 is thus compared—on a log scale—in cultures with 2 ME and cultures without 2 ME. The marked differences represent further stimulations caused by 2 ME above that seen in the corresponding MLR in the absence of 2 ME.

The ten different MLRs react differently to the added 2 ME, but in each one, stimulation is seen at least in some part of the graphs. For the sake of clarity the graphs are shown in pairs with similarities in curve form the members of the pairs are otherwise not related. It is not known whether the different behaviour to exposure to 2 ME is a characteristic of the tested individuals or whether variations in experimental set-up cause the variability The error of each $\log_{10}$ difference amounts to approximately 0.21 units.—In three of the MLRs 2 ME increased

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EFFECT OF 2-MERCAPTOETHANOL ON THE MIXED LEUKOCYTE REACTION IN MAN

Probable Effect on Blastogenic Factor Stabilization

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added at the beginning of the cultures, it produced only a weak effect when it was added after a few days culture, the effect was strong, although variable. This is probably due to a protection or activation of a soluble product, a blastogenic factor

MATERIALS AND METHODS

For details of the MLR technique see (10) Leucocytes were separated by dextran from heparinized blood taken from healthy subjects. One-way MLR was obtained by pretreatment of leukocytes from one donor in pair (stimulator) with mitomycin C Each culture tube contained 1.5 ml of medium—30 per cent heat inactivated normal human serum in Parker 199 with heparin and antibiotics added—and 3×10^6 cells Half of the cells were stimulator cells, half were non-mitomycin C treated responder cells. Triplicate tubes were used. The cells were cultured for 7 days at 37 C.

In some experiments, the supernatant from MLR cultures was used as a source of blastogenic factor For such cultures, 100 ml Erlenmeyer flasks were often used Cells were removed by centrifugation at 200 *G* for 5 minutes followed by second centrifugation at 1000 *G* for 10 minutes The medium was usually used fresh, but sometimes it was stored at −80° C without apparent loss of its blastogenic activity The blastogenic effect of the medium was tested on cells from one individual combined with mitomycin treated cells from the same individual (mtA + A) These cultures were run for 2–4 days the medium was then changed to the medium to be tested for blastogenic factor activity and the cultures were continued until day 7

2 ME (Serva Heidelberg) was diluted with saline to 1 1000 stock solution kept at +4 C up to 4 weeks Just before use, the stock solution was diluted with saline (1.20) and 50 µl of that solution was added to 1 5 ml of culture medium, giving final concentration of $\sim 7 \times 10$ M

DNA synthesis was evaluated as described by *Hedberg et al* (10) After one hour incubation with tritiated thymidine (methyl-³H-thymidine, Schwarz/Mann, 1 9 Ci/mM, final concentration 1 0 µCi/ml) nucleosides and nucleotides were extracted with cold trichloroacetic acid and the residue was dissolved in Soluene 350 (Packard Ltd) and mixed with scintillation fluid. Radioactivity was assayed by 10 minutes counting in Packard TriCarb liquid scintillator with external standardization I order to obtain near-normal distribution of the counts (*cf* 10) cpm were expressed in $\log_{10}$ form Stimulation in culture was thus evaluated as the difference between $\log_{10}$ cpm in "positive culture (mtA + B) and in control" (mtB + B) the effect of addition of blastogenic factor containing media or of 2 ME was evaluated in a similar way as $\log_{10}$ differences between experimental group and corresponding control group.

RESULTS

All MLR cultures studied gave strong thymidine incorporation at day 7—stimulation index (compared with non-mixed cells) was usually around ten, but some were weaker (minimum four) and some were stronger (maximum twenty) Addition of 2 ME at the beginning of the MLR cultures results, on the average, in a very slight stimulation of thymidine incorporation at day 7 Preliminary studies using 18 combinations of cells showed that the mean $\log_{10}$ difference between the thymidine incorporation in MLRs treated with 2 ME and identical cultures without 2 ME was 0.15 ± 0.02, representing approximately 40 per cent increase in registered counts.

Subsequently we studied the effect of 2 ME when added at different times during the culture period. 2 ME was left in the MLRs until the end of the culture period at day 7 Fig 1 gives the individual results of ten such experiments and a graph showing the mean effect at different days of the addition of 2 ME. Thymidine incorporation at day 7 is thus compared—on a $\log_{10}$ scale—in cultures with 2 ME and cultures without 2 ME. The marked differences represent further stimulations caused by 2 ME above that seen in the corresponding MLR in the absence of 2 ME.

The ten different MLRs react differently to the added 2 ME, but in each one, stimulation is seen at least in some part of the graphs. For the sake of clarity the graphs are shown in pairs with similarities in curve form the members of the pairs are otherwise not related. It is not known whether the different behaviour to exposure to 2 ME is a characteristic of the tested individuals or whether variations in experimental set-up cause the variability The error of each $\log_{10}$ difference amounts to approximately 0.21 units.—In three of the MLRs 2 ME increased

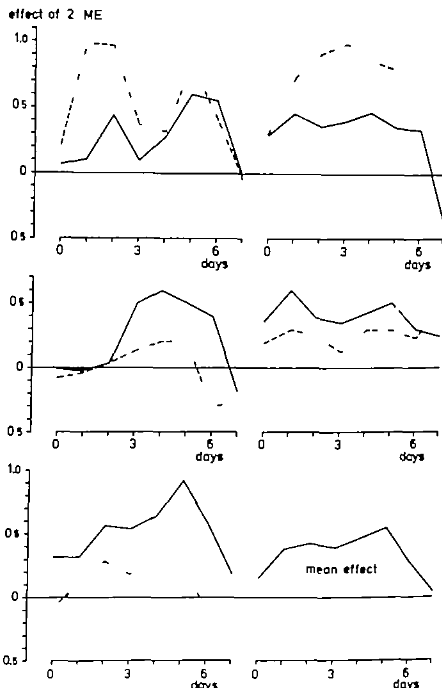


Fig 1 Effect of 2 ME on MLRs between cells from healthy subjects. Abscissa shows culture day when 2 ME was added, and ordinate shows the difference between thymidine incorporation expressed as $\log_{10}$ c.p.m. in cultures with 2 ME added and $\log_{10}$ c.p.m in corresponding cultures without 2 ME. Each line represents one cell combination. The graph in the lower right figure shows the means of all experiments.

thymidine incorporation to give a $\log_{10}$ difference of approximately 1.0 (0.92–1.00) i.e. a stimulation index of nearly ten. In six MLRs, the $\log_{10}$ differences of approximately 0.5 (0.45–0.60) were reached, i.e. a further stimulation of approximately three to four times and in one MLR, a $\log_{10}$ difference of only 0.21 was reached, i.e. approximately 60 per cent increase in thymidine incorporation above that seen in the MLR *in the* absence of 2 ME. The graphs also show that some experiments demonstrated a clear bi phasic effect, an early peak being obtained when 2 ME was added after one or two days

of incubation and a later peak when 2 ME was added after four or five days incubation. Other graphs show only the latter peak still others show approximately the same degree of stimulation through days 1 to 6. In most experiments, the addition of 2 ME during the last hour of culture (2 ME was added simultaneously with the tritiated thymidine) gave no enhancement but resulted instead in a slight depression of thymidine incorporation

The relatively late effect of 2 ME in the cultures suggested that its action was not on the initial, macrophage-dependent phase, but on a later phase where blastogenic factor (BF) perhaps plays a significant role A series of experiments was therefore made in order to study the effect of 2 ME on BF Fig 2 shows the effects of the BF containing media. The target cells were a combination of mitomycin C treated and mitomycin C non-treated cells from the same individual (mitA + A) Media added were taken from cultures of cell mixtures of mitomycin treated cells from this subject and non-treated cells from another subject (mitA + B)

As controls, media from combinations mitA + A or from mitB + B were used. Fig 2 shows that the effect of different media from mitA + B cultures varied considerably a maximum effect corresponding to a log differ ence of 1 41 which represents a 25-fold increase in thymidine incorporation, and a negligible minimum effect ($\log_{10}$ difference = 0.05 12 per cent increase) The effect of media from the control cultures varied from +0 33 to −0 70 (mitA + A) and from +0 46 to −0 44 (mitB + B) The mean effect of the mitA + B media is 0.65 ± 0.09 whereas the mean effect of the control media (both groups) is −0.04 ± 0 04

Table 1 summarizes the effect of addition of 2 ME at a concentration of 2.7 × 10 M to various steps. Five different experimental groups were studied.

In one group, 2 ME was added for 3 hours to cultures that produced BF before the removal of the medium. An enhancement of the BF effect was seen in media from mitA + B cultures (positive media) with a mean effect of 0.49 ± 0 13 No 2 ME effect on control media (from mitA + A or mitB + B) was seen

In the second group, media were taken from the producing cultures, 2 ME was added, and the media were incubated for 3 hours at 37 C and then added to the

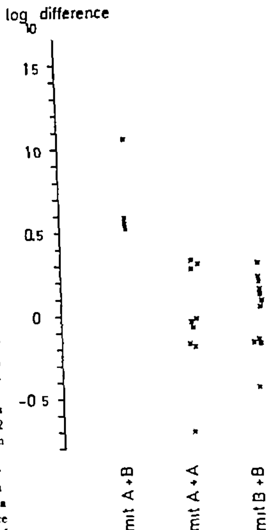


Fig 2 Effect of transferred blastogenic factor in media three types of cultures mitA + B (= positive culture) mitA + A and mitB + B (controls) when tested on cultures of mitA + A. Ordinate gives difference as $\log_{10}$ p m between cultures where the medium was changed at days 2, 3 or 4 and identical cultures without change of medium

TABLE 1 *Effect of 2 ME Addition Recorded as Log_{10} Difference between Cultures with and without Added 2 ME Medium Change at Day 2 3 or 4 Culture Time of Target Cells Days*

Experimental group	Number of experiments	Mean effect of 2 ME ± S.E.M.	t
2 ME added 3 h before removal of medium from BF producing cultures			
mitA + B cultures	12	0 49 ± 0 13	3 77*
mitA + A or mitB + B cultures	13	0 00 ± 0 07	0
2 ME added to removed medium and incubated for 3 h, 37 C and then added to the target cultures			
mitA + B medium	9	—0.10 ± 0 05	—2.0
mitB + B medium	6	—0 05 ± 0 12	—0.42
2 ME added to media just before addition to reactor cells			
mitA + B medium	6	0.28 ± 0 04	7.00***
mitA + A or mitB + B medium	6	—0 02 ± 0 10	—0.20
2 ME added to reactor cells 3 h before change of medium			
mitA + B medium	5	0 08 ± 0.13	0.62
mitA + A medium	5	0 03 ± 0 10	0.30
2 ME added to reactor cells after change of medium			
mitA + B medium	9	0.11 ± 0 07	1.57
mitB + B medium	6	—0 08 ± 0 09	—0.89

target cultures. No effect of the 2 ME treatment was seen.

In the third group 2 ME was added to the media just before they were added to the target cells. A clear-cut enhancement of the effect of the positive medium (from mitA + B cultures) was then registered, but no effect on media from control cultures (mitA + A or mitB + B) was seen

In the fourth group 2 ME was added to the taget cells 3 hours before the addition of the BF-containing media—no effect was seen

In the fifth group 2 ME was added to the reactor cells after the medium change i e after the transfer of the BF to the target cells. No effect of 2 ME was seen on the average but in the 9 experiments with medium from mitA + B cultures, enhancement amounting to from +0 25 to +0 38 was recorded in 4 experiments, but this could be random

The difference between Groups 3 and 5 is small. In Group 3 2 ME was added to the medium just before addition of the medium to the target cells in Group 5 after addition of the medium none the less, a definite effect was seen only in Group 3

DISCUSSION

When 2 ME ($2 7 \times 10^{-5}$ M) was added at the beginning of the MLR cultures, the effect was weak thymidine incorporation was increased by an average of 40 per cent. 2 ME increased incorporation with a factor of 4 7 (final 2 ME concentration was 6×10^{-4} M) in MLRs between mouse spleen cells freed of adherent cells (1) When 2 ME was added at $2 5 \times 10^{-4}$ M at the beginning of MLRs between mouse spleen cells in a protein-free medium, *Heber Katz & Click* (9) found a 30-fold increase in thymidine incorporation In those studies however 2 ME was added to a culture system that, without 2 ME, showed a very low proliferative response due to lack of adherent cells or lack of protein in the culture medium. In our series, the MLRs showed strong thymidine incorporation also in the absence of 2 ME.

Heber Katz & Click (9) found that the addition of 2 ME after 12 hours culture gave a lower enhancement than addition at the beginning of the culture when 2 ME was added after 24 hours, no effect was seen. They suggest that a 2 ME-sensitive "state" or "entity" exists with a life-span of less than 24 hours, and that it is destroyed in the absence of 2 ME. The total culture time in their experiments was, at most, 60 hours. On the other hand, *Engers et al.* (7) reported that they obtained effects on the formation of cytotoxic effector cells in MLR when 2 ME was added at day 3 of a culture as well as at the beginning and *Soulillou et al* (17) obtained better effects if 2 ME was added during the first 48 hours of culture but could demonstrate an effect also at days 4 or 5. We found human MLRs relatively insensitive to 2 ME during the initial culture period, but much more sensitive several days after culture began. The action time of added 2 ME in our experiments is probably quite short. This is evident already from Fig 1 Addition of 2 ME at day 3 for instance sometimes gave only a weak reaction, but added at day 4 it could produce a marked stimulation. The experiments with blastogenic factor-containing media resulted in similar experiences 2 ME added to the medium 3 hours before its addition to the target cells, or added to the target cultures 3 hours before the addition of medium, produced no effect in the same experiments where fresh 2 ME added to the medium resulted in strong enhancement. Apparently 2 ME activity is completely abolished within 3 hours or perhaps less under culture conditions. Probably the active radical of 2 ME is the sulphydryl group which can easily be oxidized under experimental conditions, but *Broome & Jeng* (2) point out that the formed disulphide can enter the cell and then again be easily reduced, and stimulation of the cytotoxic response in MLR could be obtained also with oxidized 2 ME, according to *Engers et al.* (7)

In the present study the observations on the action of 2 ME, in experiments where the effect of blastogenic factor (BF) was tested, suggest that 2 ME acts in some way on this soluble component in the MLR A very marked enhancement of BF effect could be found in two experimental situations when 2 ME was added for three hours during the production of BF and when 2 ME was added together with BF to the target cells. As a relatively good effect was obtained with 2 ME when added after the separation of BF from the producing cells, it is unlikely that 2 ME acts by increasing the production of BF More probably it protects BF from degradation in a cellular system. It has no effect on degradation of BF when incubated free from cells for three hours, but it enhanced the effect of BF in the presence of cells, in either the producing culture or the reacting cultures. This argues in favour of a protective mechanism rather than a potentiating mechanism. Perhaps 2 ME increases the amount of transferred BF when added during the last 3 hours of production by reducing the breakdown of BF caused by products from the living cells. Similarly 2 ME might protect the amount of BF transferred for a certain time against breakdown and thus increase the number of target cells affected by BF This explanation resembles that given by *Melsom et al.* (13) concerning the stabilizing effect of 2 ME on the labile macrophage cytolytic factor Perhaps the effect of 2 ME in many *in vitro* immunological systems is that of protecting small amounts of soluble factors from destruction and in this way apparently substituting for the cells producing the factors, *e.g* macrophages. *Metcalf's* (14) recent results on the effect of 2 ME on B lymphocyte colony formation *in vitro* can also be interpreted in this way

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REFERENCES

1 *Bevan M J Epstein R. & Cohn M* The effect of 2-mercaptoethanol on murine lymphocyte cultures. J exp. Med. 139 1025–1030 1974

2. *Broome J D & Jeng M W* Promotion of replication in lymphoid cells by specific thiols and disulfides *in vitro* J exp Med. *138* 574–592 1973
3 *Chen C & Hirsch J G* The effects of mercaptoethanol and of peritoneal macrophages on the antibody-forming capacity of non-adherent mouse spleen cells *in vitro* J exp. Med. *136* 604–617 1972
4 *Chen C & Hirsch J G* Restoration of antibody-forming capacity in cultures of non-adherent spleen cells by mercaptoethanol Science *176* 60–61 1972
5 *Click R E. Benck L. & Alter B J* Enhancement of antibody synthesis *in vitro* by mercaptoethanol. Cell. Immunol *3* 156–160 1972
6 *Daniele R P & Rowlands D T Jr* Detection of human lymphocytes bearing complement receptors after *in vitro* modification with mercaptoethanol. Transplantation *17* 126–128 1974
7 *Engers H D MacDonald J H R., Cerottini J-C & Brunner K T* Effect of delayed addition of 2 mercaptoethanol on the generation of mouse cytotoxic T-lymphocytes in mixed leukocyte cultures. Europ J Immunol *5* 223–225 1975
8. *Fanger M W Hart D A Wells J V & Nisonoff A* Enhancement by reducing agents of the transformation of human and rabbit peripheral lymphocytes. J Immunol *105* 1043–1045 1970
9 *Heber Katz E & Click R E* Immune response *in vitro* V Role of mercaptoethanol in the mixed leukocyte reaction. Cell. Immunol. *5* 410–418 1972
10 *Hedberg H Källén B Löw B. & Nilsson, O* Impaired mixed leukocyte reaction in some different diseases, notably multiple sclerosis and various arthritides. Clin. exp Immunol *9* 201–207 1971
11 *Katz Heber E., Peck A B. & Click R E.* Immune response *in vitro* II Mixed leukocyte interaction in a protein-free medium. Europ J Immunol. *3* 379–385 1973
12 *Lafferty K Ryan M & Misko I* An improved system for the assay of stimulation in mouse mixed leucocyte cultures. J Immunol. Methods *4* 263–273 1974
13 *Melsom H Sanner T & Seljelid R* Macrophage cytolytic factor Some observations on its physicochemical properties and site of action. Exp. Cell. Res. *94* 221–226, 1975
14 *Metcalf D.,* Role of mercaptoethanol and endotoxin in stimulating B lymphocyte colony formation *in vitro* J Immunol *116* 635–638 1976
15 *Rode H N & Gordon J.,* Macrophages in the mixed leukocyte culture reaction (MLC) Cell Immunol. *13* 87–94 1974
16 *Rosenstreich D L. & Wilton J M* The mechanism of action of macrophages in the activation of T-lymphocytes *in vitro* by antigens and mitogens. In Rosenthal A. S. (Ed.) Immune recognition. Proc. IX Leukocyte Culture Conference. Academic Press, New York 1975 p 113–132
17 *Soulillou J P Carpenter C B Lundin A P & Strom T B* Augmentation of proliferation and *in vitro* production of cytotoxic cells by 2 ME in the rat. J Immunol. *115* 1566–1571 1975

Acta path. microbiol. scand. Sect. C, 84: 397–402, 1976

FURTHER CHARACTERIZATION OF PROTEIN A REACTIVE AND NON-REACTIVE SUBFRAGMENTS OF Fc FROM HUMAN IgG

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Endresen, C. & Grov, A. Further characterization of protein A reactive and non-reactive subfragments of Fc from human IgG. Acta path. *microbiol.* scand. Sect. C, *84*: 397–402, 1976.

Tryptic digests of acid-treated Fc from normal human IgG were separated into four peaks (I–IV) by gel filtration on Sephadex G-100. The second peak was *further* divided into two fractions (II and II'). Peak I was indistinguishable from intact Fc on electrophoresis, immunodiffusion, and reactivity to protein A. The protein A reactive fragments of fractions II, II' and III were shown to *contain* antigenic determinants of both the C_H2 and C_H3 domains, to interact with the anti-Gm (1) specific rheumatoid factor and to fix complement. These results, together with SDS-electrophoresis, showed that protein A reactive *fragments are all* composed of an intact Fc chain with shorter chains covalently linked to it. The protein A non-reactive fragments of fractions II' and III were homogeneous, fixed complement and showed no interaction with the Gm (1) rheumatoid factor. These results, in addition to the observed antigenic determinants, localized the fragments to the C_H2 region.

Key words: Fc subfragments; human IgG; protein A *interaction*.

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Several subfragments belonging to the C_H3 homology region of the Fc have been isolated and characterized, whereas *subfragments* from the N-terminal half of the Fc (the C_H2 region) have proved difficult to isolate. The C_H2 region is known to undergo extensive degradation during production of Fc and pFc fragments.

Ellerson et al. (4) have described a method in which acid treatment of the Fc *fragments* of *human* IgG, prior to short trypsin digestion, resulted in isolation of subfragments corresponding to the C_H2 and C_H3 regions. In a previous paper (5) we reported the results of the application of this procedure to Fc from pooled *human* IgG. The fragments, being separated into 4 peaks on gel filtration were examined for interaction with staphylococcal protein A. Reactive material was found in peaks I–III. The protein A reactive material from peaks II and III was found to be composed of an intact Fc chain and a shorter one, amputated on the C-*terminal* side. Furthermore, electrophoretic and serological examinations indicated that the protein A non-reactive fragments of peak III originated from the C_H2 region.

The purpose of the present study was to characterize more closely the protein A reactive fragments and the non-reactive fragments of peak III.

2. *Broome J D & Jeng M W* Promotion of replication in lymphoid cells by specific thiols and disulfides *in vitro* J exp Med. *138* 574–592 1973

3 *Chen C & Hirsch J G* The effects of mercaptoethanol and of peritoneal macrophages on the antibody-forming capacity of non-adherent mouse spleen cells *in vitro* J exp. Med *136* 604–617 1972.

4 *Chen C & Hirsch J G* Restoration of antibody forming capacity in cultures of non-adherent spleen cells by mercaptoethanol. Science *176* 60–61 1972

5 *Click R E. Benck L. & Alter B. J* Enhancement of antibody synthesis *in vitro* by mercaptoethanol. Cell. Immunol *3* 156–160 1972

6 *Daniels R P & Rowlands D T Jr* Detection of human lymphocytes bearing complement receptors after *in vitro* modification with mercaptoethanol Transplantation *17* 126–128, 1974

7 *Engers H D MacDonald J H R Cerottini J C & Brunner K T* Effect of delayed addition of 2 mercaptoethanol on the generation of mouse cytotoxic T-lymphocytes in mixed leukocyte cultures. Europ J Immunol *5* 223–225 1975

8 *Fanger M W Hart D A Wells J V & Nisonoff A* Enhancement by reducing agents of the transformation of human and rabbit peripheral lymphocytes. J Immunol. *105* 1043–1045 1970

9 *Heber Katz E & Click R E.* Immune response *in vitro* V Role of mercaptoethanol in the mixed-leukocyte reaction Cell Immunol. *5* 410–418 1972

10 *Hedberg H Källén B. Löw B. & Nilsson, O* Impaired mixed leukocyte reaction in some different diseases, notably multiple sclerosis and various arthritides. Clin. exp. Immunol. *9* 201–207 1971

11 *Katz Heber E. Peck A B. & Click, R. E.* Immune response *in vitro* II Mixed leukocyte interaction in a protein-free medium. Europ J Immunol *3* 379–385 1973

12 *Lafferty K Ryan M & Misko I* An improved system for the assay of stimulation in mouse mixed leucocyte cultures. J Immunol. Methods *4* 263–273 1974

13 *Melsom H Sanner T & Seljelid R.* Macrophage cytolytic factor Some observations on its physicochemical properties and site of action Exp Cell Res. *94* 221-226 1975

14 *Metcalf D* Role of mercaptoethanol and endotoxin in stimulating B lymphocyte colony formation *in vitro* J Immunol *116* 635-638 1976

15 *Rode H N & Gordon J* Macrophages in the mixed leukocyte culture reaction (MLC) Cell. Immunol *13* 87–94 1974

16 *Rosenstreich D L. & Wilton J M* The mechanism of action of macrophages in the activation of T-lymphocytes *in vitro* by antigens and mitogens. In Rosenthal A. S. (Ed.) Immune recognition. Proc. IX Leukocyte Culture Conference Academic Press, New York 1975 p 113–132.

17 *Soulillou J P Carpenter C B. Lundin A P & Strom T B.* Augmentation of proliferation and *in vitro* production of cytotoxic cells by 2 ME in the rat. J Immunol. *115* 1566–1571 1975

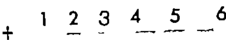


Fig. 2. SDS polyacrylamide gel electrophoresis of fragments II+ (1), II'+ (2), III+ (3), II— (4), II'— (5) and III— (6).



Fig. 4. Double diffusion in agar gel. The wells in A, C, and E were filled with Fc (1), II+ (2), II'+ (3), III+ (4), pFc' (5) and peak IV (6), the center wells with anti-Fc, anti-pFc' and anti-C_H2, respectively. The wells in B, D and F were filled with Fc (1 and 4), III— (2), peak IV (3), pFc' (5) and II— (6) and the center wells with anti-Fc, anti-pFc' and anti-C_H2, respectively.

alkylated III+ moved almost as prior to reduction, suggesting that III+ has a very short piece covalently attached to the intact chain. Reduced and alkylated II— and III— both moved similarly as myoglobin (mol wt. = 17,000), showing shorter chains than that of intact Fc (Fig. 3).

Fig. 4 (A–D) shows the results of agar diffusion of the fragments against anti-Fc and anti-pFc' sera. The protein A reactive fragments (Fc, II+, II'+, III+) gave a reaction of identity with both sera (Fig. 4 A and C). The peak IV and pFc' fragments, behaving as identical substances against both antisera, gave a reaction of partial identity with the protein A reactive fragments against anti-Fc, and a reaction of identity against anti-pFc' serum. The non-reactive fragments (II'— and III—) did not react with anti-pFc' serum, gave a reaction of partial identity with Fc and non-identity with peak IV fragments against the anti-Fc serum (Fig. 4 D and B). Using a specific C_H2 antiserum (anti Fc absorbed with pFc' fragments), the protein A reactive fractions showed a reaction of identity (Fig. 4 E); peak IV and pFc' fragments did not precipitate, whereas both II'— and III— still reacted (Fig. 4 F). In immunoelectrophoresis, fragments II+, II'+ and III+ gave single precipitation lines with almost identical mobility (Fig. 5). Fragment II— appeared rather heterogeneous, in accordance with SDS-electrophoresis. Fragments II'— and III— both gave one precipitin line against anti Fc serum, the position

+ 1 2 3 4 5 6 7 8

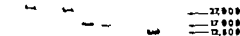


Fig. 3. SDS polyacrylamide gel electrophoresis of reduced and alkylated fragments II+ (1), II'+ (2), III+ (3), II'— (4) and III— (5). Myoglobin (mol wt. 17,000) (6), cytochrome C (mol wt. 12,500) (7) and reduced and alkylated Fc fragments (mol wt. 27,000) (8) were run as marker proteins.

MATERIALS AND METHODS

Human IgG and Fc fragments. The Cohn fraction II preparation of pooled human IgG (Kabi AB Sweden) was used and Fc fragments prepared as described in (15) By this method mainly Fc of IgG_1 is obtained. The papain used was from Sigma (USA) (2 × crystallized from Papaya latex) The Fc fragments were tested for purity by double dif fusion in agar and immunoelectrophoresis using specific antisera and by polyacrylamide gel elec trophoresis.

Fragmentation of Fc The trypsin digestion of Fc and gel filtration of the digest were carried out as before (4 5) Further purification of the fractions was performed by re-cycling on a column (2.5 × 120 cm) of Sephadex G-100 superfine in 1 N HAc and a column (1 6 × 70.5 cm) of Sephadex G-200 in 5 M guanidine – 1 M HAc The K values of the proteins were calculated (2)

Proteins were concentrated by ultrafiltration using Amicon cells and UMO5 and UM2 filters (Diaflo) and quantified as described in (11)

Reduction and alkylation was performed (16) using a protein concentration of 5–10 mg per ml

Apparent molecular weight was determined (8) using the K_a values of the proteins together with those estimated for blue dextran (Pharmacia, Sweden) human serum albumin (Sigma) oval bumin (Boehringer Mannheim Germany) cyto-chrome C (Sigma) and insulin (Sigma)

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out (6) in 11.2 per cent gel and 1 per cent SDS using a Bio-Rad Model 220 apparatus, and both *immunoelectrophoresis and double diffusion in agar* were per formed as before (5)

Sepharose protein A immunoadsorbent was prepared as described in (7)

Antisera to human serum, IgG and Fc frag ments were produced as described earlier (4) An anti pFc serum was kindly provided by Dr T E Michaelsen Inst Immunol Rheumatol Un versity Hospital, Oslo Norway

Haemagglutination inhibition studies using hu man ORh+ (R R_2) erythrocytes and anti D/Gm (1) serum (Behringwerke, BRD) were carried out as in (9)

Complement fixation assay The ability of Fc fragments to fix guinea pig complement was tested using polystyrene particles (Difco USA) (14) the amount of CH_{50} units being estimat d from the intercepts of von Krogh plots (13)

Carbohydrate determination Samples of protein were hydrolysed in 4 N HCl for 3 h and the pre sence of hexosamines examined (12)

N and C-terminal amino acids Samples (1 3 mg) were digested with leucine aminopeptidase (Sigma) (10) and carboxypeptidase A (Sigma) (1) the enzyme to substrate ratio in both cases being 1 25 Aliquots were removed from digests after 4 16 and 48 h and tested for free amino acids (17) N terminal amino acids were also examined by the dansylation method (18)

RESULTS

Gel filtration of the tryptic digest of Fc fragments revealed four peaks (Fig 1) peak II being further divided into two fractions. The five fractions, further purified on Sephadex G 100 superfine were examined for protein A interaction on Sephadex protein A columns and all except peak IV contained both reactive (+) and non reactive (—) portions. *Most of the material in peak I was protein A* reactive and found to be undigested Fc frag ments Peak IV fragments, being completely non reactive to protein A have been shown to originate from the C_H3 region (4 5) Re peated acid treatment tryptic digestion, and gel filtration of II+ fragments gave 30-40 per cent of intact material (II+) and frag ments corresponding to peaks III and IV

SDS-polyacrylamide gel electrophoresis of the isolated subfragments (Fig 2) showed that II+ and II + contained a main frag ment and traces of two or three other frag ments. III+ II'— and III— seemed rather homogeneous, whereas II— contained two major and two minor fragments. Electro-phoresis of reduced and alkylated samples (Fig 3) indicated that an intact Fc chain as well as a shorter piece were present in all protein A reactive fragments. Reduced and

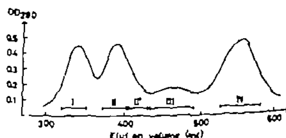


Fig 1 Gel filtration on Sephadex G-100 of a tryptic digest of acid-treated Fc from normal human IgG The column (3.6 × 87.5 cm, V = 259 ml) was eq librated and eluted with 1.0 M acetic acid containing 25 mM NaCl (elution rate 16 ml per h)

mhibited due to shortage of material, but II+ II'+ and III+ showed mainly glycine as C-terminal.

DISCUSSION

Normally Fc fragments of human IgG are rather resistant to proteolytic enzymes, but acid treatment apparently renders such fragments susceptible to trypsin (4 5) The use of Fc fragments from pooled normal IgG (5) seemed to result in a somewhat more complex digest than that of IgG (4) Also in the present study the Fc fragments were predominantly from the IgG subclass (15) Preliminary experiments indicated that peak II (Fig. 1) being the most heterogeneous one should be divided into two fractions (II and II') so as to obtain a better separation and purification. Fractions II, II' and III contained protein A reactive (+) fragments, all of which were shown to contain antigenic determinants of both the C_H2 and C_H3 domains (Fig 4) Low molecular weight fragments showing identity to the C_H3 domain were split off by further digestion of acid treated fraction II In addition, all the protein A reactive fragments interacted with a rheumatoid factor with anti-Gm (1) specificity and fixed complement, these properties being localized to the C- and N-terminal, respectively These results, together with the SDS-electrophoresis, point to the conclusion that the II+ II'+ and III+ fragments are composed of an intact Fc chain with shorter chains of different length covalently linked to it (Fig 7) In fragment III+ such an extra piece must be very short since no clear difference in electrophoretic mobility was observed before and after reduction.

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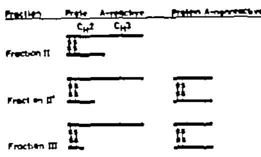


Fig 7 Proposed structure of the Fc subfragments from peaks II and III

REFERENCES

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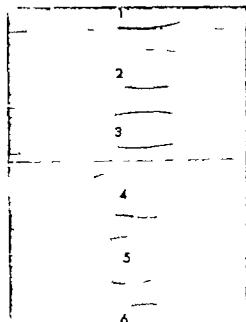


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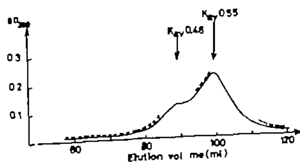


Fig 6 Gel filtration on Sephadex G-200 of reduced and alkylated protein A non reactive fragments (II — and III—) The column (1 6 × 70 5 cm V_o = 44 ml) was equilibrated and eluted with 5 M guanidine – 1 M acetic acid (elution rate 3.5 ml per h)
—— red & alk. III— ----- red & alk. II —

TABLE 1 *Haemagglutination Inhibition of the System Anti Gm (1) Antibodies and Human O Red Blood Cells Coated with Anti D/Gm (1) Antibodies*

Fragment	Inhibiting dose (mg)
Fc	0 125–0 250
II +	0.5
III +	0.5
II —	2.0–4.0
III—	2.0–4 0
Fab	>10 0

a main peak and a minor one with K_{av} values corresponding to molecular weights of about 16 000 and 24 000 respectively (Fig 6) The material in the main peak reacted with anti Fc and anti C_H2 sera, but not with anti-pFc serum and was apparently pure C_H2 fragments. The material of the minor peak reacted with all of the three antisera.

In the haemagglutination inhibition test, all the protein A reactive fragments were effective So were peak IV fragments in contrast to II— and III— fragments (Table 1) In the complement fixation test however peak IV (C_H3) fragments were nearly negative whereas II — III— and other fragments containing the C_H2 structure were positive (Table 2)

All fragments, except peak IV contained hexosamine F(ab)₂ and Fc fragments were included as a negative and positive control, respectively

The N terminal amino acid analysis indicated threonine in that position in all protein A reactive fractions as well as in III— fragments The C-terminal analysis was partly

TABLE 2 *Complement Fixation Capacity of Human IgG Fc and Subfragments of Fc (CH Units Fixed per 50 µg Protein)*

Sample	Experiments 1	2	3	4
γG	15 ?	11 7	13 2	14.8
Fc	9 1	8.8	11 1	12 7
peak III— (Cγ2)	8 8	5.5	7.2	9.8
peak IV (Cγ3)	2 1	1 1	2.2	1.8

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DISCUSSION

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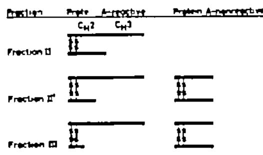


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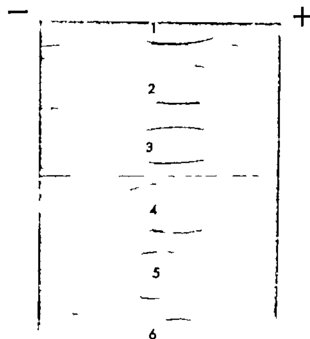


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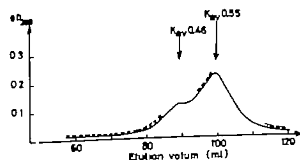


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Acta path. microbiol. scand. Sect. C, 84: 403–413, 1976

GENERAL IMMUNOCOMPETENCE AND TUMOUR-DIRECTED CELL MEDIATED HYPERSENSITIVITY *IN VITRO* IN PATIENTS WITH RENAL CARCINOMA

MOGENS KJAER and MOGENS THOMSEN

Division of Clinical Immunology, Medical Department TA, and Surgical Department C, Rigshospitalet, Tissue Typing Laboratory, Rigshospitalet, University Hospital of Copenhagen, Denmark

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Thirteen patients with renal carcinoma were examined for tumour-directed, cell-mediated hypersensitivity (TCMH) by means of the leucocyte migration technique, and for general immunocompetence (GIC) by means of quantitation of T- and B-cells in peripheral blood and studies of lymphocyte transformation in vitro using a panel of antigens and mitogens. Eight out of 13 patients had evidence of TCMH, six out of 13 had abnormal GIC. Any correlation between the presence of TCMH and normal/abnormal GIC was not found. There was a trend towards a positive correlation between the absence of distant metastases and evidence of TCMH. If both TCMH and GIC were considered, significant correlation between the presence of distant metastases, lack of TCMH and/or abnormal GIC was demonstrated. It is concluded that the defect TCMH usually found in patients with renal carcinoma and disseminated disease cannot be explained exclusively by defects in GIC.

Key words: Renal carcinoma; immunocompetence; tumour-directed, cell-mediated hypersensitivity.

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Numerous studies of patients with solid tumours have been published in the last few years concerning total lymphocyte counts in peripheral blood (28), the proportion of T- and B-cells (2, 7, 9, 11, 27), and the ability of lymphocytes to respond to common mitogens and antigens in vitro (1, 4, 5, 6, 8, 10, 20, 21, 23, 24, 25, 29, 30, 31, 32, 33). Data obtained by these parameters will in the following be termed the general immunocompetence (GIC).

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3 GIC Controls

The control series participating in the investigation of GIC was composed of technicians and doctors in the Tissue Typing Laboratory Rigshospitalet, and blood donors selected as mentioned above, in all 37 persons. Sixteen males and 1 females participated. Mean age 30 (range 15–58) years. This series has been described in detail elsewhere (26)

Preparation of Tissue Ext act from Renal Carcinoma and Corresponding Normal Renal Tissue Together with Description of the Capillary Tube Leucocyt Migration Technique (LMCT)

The preparation of tissue extracts from renal carcinoma and corresponding normal renal tissues has earlier been described in greater detail (15 1). Only non-pooled extracts from allogeneic renal carcinomas and renal tissues were used.

The micromodified LMCT was performed according to previous description (15, 16). In essence, venous blood was collected in heparin and dextran. After sedimentation, the leucocytes were washed and collected in capillary tubes. Each of these was placed in 0.5 ml disposable migration chamber containing TC 199 with 10 per cent horse serum. Tissue extract was added in relevant concentrations to the test cultures. All cultures were set up in triplicate or quadruplicate. After 24 hours, the migration area of leucocytes around the opening of the capillary tube was measured in a projection microscope. The average area of migration of extract-containing (M_X) cultures and control (M_0) cultures from one and the same blood sample determines the migration index (MI) MI M_X/M_0.

Using three leucocyte washings, dose-related effect of tumour extract on the in vitro migration of leucocytes from patients with non-metastatic renal carcinoma has earlier been shown (15 16). Using six or ten leucocyte washings, the stage-related effect of tumour extract disappears and almost all patients show reactivity (17). Since the aim of the present study was to correlate stage-related effects three leucocyte washings were employed.

Lymphocyte T ansformation

Blood was drawn into equal olumes of RPMI 1640 ith heparin. The mononuclear cells were isolated on Lymphoprep (Nyegaard, Oslo) gradient.

According to some studies (6 23 24) lack of response to PHA in cancer patients might be attributed to 1) an intrinsic lymphocyte defect or 2) the presence of inhibiting activity in sera f om cancer patients. T avoid the effect of serum-mediated inhibition, homologous serum w used in all transformation studies. The cells were washed thrice in RPMI 1640 with 5 per cent pooled human serum from non-transfused young male donors. The cultures were set up in tubes w th 10^5 cells in 0.5 ml of RPMI 1640 with 15 per cent serum. To the medium was added 250 U/ml penicillin and 200 U/ml streptomycin, 1.2 mM glutamine and 25 mM Hepes.

After addition of mitogens and antigens in various concentrations, the cells were cultured in 5 per cent CO in humidified air at 37 C for 96 hours (mitogen stimulated cultures) or for 120 hours (antigen stimulated cultures)

All cultures were made in triplicate

Twenty-four hours before termination of cultures, 0.05 µl ^{14}C-thymidin was added to each tube. The cell were harvested on glass-fibre-filters using Skatron (Oslo, Norway) semiautomatic harvesting machine, followed by washing with distilled w ter. The filters were placed in counting ials and 5 ml of Instagel® was added before liquid scintillation counting. The stimulation was expressed as counts per minute (cpm) in stimulated cultures minus cpm in unstimulated cultures. The median cpm of the triplicates was used for calculations.

Mitogens and Antigens

Phytohaemagglutinin (PHA P Difco) in a dilution of 1:600 was added, pokeweed mitogen (PwM, Gibco) being added in a dilution of 1:2500.

Heat killed *S aureus* (SA) and *E coli* (EC) were kindly prepared by *Klaus Jensen* and used at concentrations of 5 × 10 and 5 × 10^4 organisms per culture

C albicans (CA) extract prepared by *Christia Thestrup* was employed in protein concentrations of 1000 and 100 µg per culture. PPD without chinosol (Statens Seruminstitut) was used in concentrations of 5 and 0.5 µg per culture tube

Rosette Forming Cells

For the E and EAC rosette assay 3 ml of a mixture of equal volumes of blood and medium RPMI 1640 w th heparin was incubated with 0.8 g carbonyl iron and 3–4 glass beads t 37 C for 20 minutes in plastic WR tube which was slowly rotated. A powerful magnet was used to separate the phagocytosing cells the remaining cells were layered on Lymphoprep and the lymphocytes were isolated. They were then washed thrice in Hanks' solution and resuspended in Hanks' at a concentration of 3 × 10^6 cells/ml.

E Rosettes

100 µl of purified lymphocyte suspension was incubated with 100 µl of a 0.5 per cent suspension

CIC in relation to the clinical stage of cancer in the patients studied (5 30 33 8 4 32 6)

As parameter of GIC, most studies have used lymphocyte transformation techniques where phytohaemagglutinin (PHA) served as stimulant (5 8 20 29 30 31 33) Other mitogens and antigens have been used such as pokeweed mitogen (PWM) streptokinase streptodornase, *Candida albicans* and purified protein derivative (PPD) (4 10 21 25 32) In some studies, a panel of mitogens and antigens have been used (4 10 25) The results of these studies have not been conclusive, some reporting that GIC in cancer patients might be defective as compared with findings in controls (11 20 31 21 10 1 23 24) others reporting stage related defects within the cancer group (7 27 2 9 33 30 8, 6 32) Concerning the tumour-directed cell mediated hypersensitivity (TCMH) in patients with renal carcinoma, *Kjaer* has recently shown by way of the leucocyte migration capillary tube technique (LMCT) (13 15) that a proportion of these patients have TCMH directed towards components of autologous and allogeneic renal carcinoma tissue

- that TCMH is present significantly more often in patients without than in patients with distant metastases (13 16)
- that the lack of demonstrable TCMH in patients with distant metastases can be at least partly explained by the presence of a factor (circulating tumour antigen?) on lymphocytes which inhibits their reactivity This factor can be removed by extensive cell washing *in vitro* (17) and can be found in serum from the patients (12)

The possibility that TCMH non reactivity in patients with renal carcinoma and distant metastases also might involve defects in GIC made it of considerable interest to compare defects in GIC and TCMH in patients with renal carcinoma.

As regards patients with renal carcinoma the purpose of the present study was

- to investigate the presence of defects in GIC as compared with control persons,
- to investigate whether possible defects in GIC could be correlated to the clinical stage and to the histopathology of the tumours,
- to investigate whether there is a correlation between absence of TCMH against allogeneic hypernephroma tissue extracts in the LMCT and defects in GIC
- to investigate whether the immunological non reactivity in disseminated disease earlier described (13 16) could be explained by defects in GIC

PATIENTS AND METHODS

The series of patient comprised the following groups

1 *Patients with Renal Carcinoma*

Thirteen consecutive patients with histologically verified renal carcinoma, 7 men and 6 women, age 46–80 mean 62 years. In 12 patients the histological diagnosis was established as hypernephroma, 1 patient had a transitional cell carcinoma of the renal pelvis. Six patients had distant metastases at the time of diagnosis. The histopathological evaluation of tumours was performed according to criteria described in detail earlier (18) Clinical and histopathological data on the 13 patients are shown in Table 1

2 *TCMH Controls*

Extensive reports on the reactivity with the relevant tissue extracts in the LMCT of leucocytes from patients in different groups of control, have earlier been published (13–16) and are not to be described in detail here. These groups comprised a) 17 patients with various benign renal diseases. b) 22 patient with various carcinomas of non-kidney origin all without detectable distant metastases at the time of test and examined before operation c) 45 patients with various non-malignant diseases, examined during hospitalisation. They did not present signs of malignant or benign tumours, hypertension, or auto-immune diseases, and had not received glucocorticoid or cytostatic drugs within the la t six months. d) 77 members of the blood donor group of Rigshospitalet, Copenhagen who on the basis of a questionnaire concerning earlier diseases were assumed to be healthy and on a set of blood analyses made before they had been accepted as blood donors.

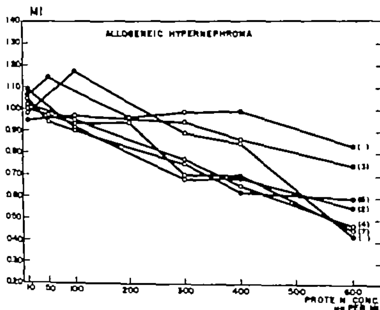


Fig. 1 Titration curves applying to 7 patients with renal carcinoma, indicating the presence of TCMH against allogeneic hypernephroma tissue extracts in the LMCT Numbers in parentheses refer to Table 1

Any significant variation in the results of these tests was not found.

The values of T and B-cells have been given as per cent of lymphocytes Lymphocyte counts in 11 out of 13 patients were available (Table 1) Using 1200 lymphocytes per µl as lower cut-off limit, no gross disturbances were observed. The values of transformation are given as increments, i.e. cpm in antigen/mitogen stimulated cultures minus cpm in unstimulated cultures.

On the basis of data obtained in the control group, a decreased GIC was defined as values below the normal range in lymphocyte transformation or in percentage of T and B-cells. The lower cut-off limit of these ranges were as follows (30) PHA>13984 cpm, PWM>3452 cpm, PPD>914 cpm, CA>953 cpm, SA>1569 cpm T-rosettes>32 per cent, B-rosettes 8–26 per cent, membrane fluorescence 8 29 per cent A grading of normal and abnormal responses is shown in Table 1

Statistical Evaluation

A computerized Fisher exact probability test and Wilcoxon Mann-Whitney test were used for statistical analysis Level of significance = 5 per cent.

RESULTS

The results of the investigation are shown in Figs. 1–4 and in Tables 1–5

Figs. 1 and 2 show LMCT titration curves applying to the 13 patients in whom allogeneic hypernephroma extracts were the antigen. No significant reactivity towards extracts of allogeneic normal renal tissue was shown. Eight out of 13 patients presented signs of TCMH (grade 2) Five out of 13 patients did not present signs of TCMH (grade 0) The transformation values and the T and B cell parameters are shown in Figs. 3 and 4

By transformation with PHA, 2 patients were found to be definitely low responders, whereas all responded normally to PWM. In three out of 13 patients, response to PPD was abnormal, in 2 out of 13 it was abnormal to CA, and in 4 out of 13 it was abnormal to SA, Fig 3. The responses to EC were omitted from the statistical evaluation because response in some of the controls was minimal. A relative decrease in number of T-cells was not seen in any of the patients (Fig 4) The two methods used in the present study for quantitation of B-cells were found to give identical results. The series of

of sheep red blood cells (SRBC) and 20 µl AB serum (absorbed with SRBC) for 30 minutes at 37 C in a Falcon plastic tube

The mixture was centrifuged at 200 G for 5 min and incubated for 18 hours at 4 C. Before counting the cell pellet was gently resuspended by rolling the tubes between the palms the counting was performed in a haemocytometer As rosettes were counted, lymphocytes with 3 or more SRBC attached to the surface. The percentage of rosette forming cells was evaluated under a light microscope at 400 × magnification. The samples were prepared in duplicate, 100 cells were counted and the mean of the two counts was determined.

EAC Rosettes

Human A erythrocytes were washed thrice in Hanks solution and resuspended at a concentration of 2.5 per cent The erythrocytes were incubated at 37 C for 30 min with an equal volume of a 1 2500 dilution of rabbit anti-A antiserum in Hanks solution The antiserum was of IgM character and obtained by immunizing a rabbit with human A erythrocytes. Before use the serum was carefully titrated to determine the optimal non agglutinating concentration.

After incubation with antiserum, 100 µl of mouse serum per 2 ml of erythrocytes suspension was added the suspension was carefully mixed and incubated for another 30 min at 37 C Finally 3 ml of Hanks solution was added In the rosette assay 100 µl of lymphocyte suspension was mixed with 100 µl of erythrocyte suspension in Falcon tubes and centrifuged for 5 min at 200 G 20 µl 0 1 per cent toluidine blue was added and, after 5 minutes the cell pellet was resuspended by rolling the tubes gently between the palms and counted as described above

Direct Membrane Immunofluorescence

Blood mixed with an equal volume of RPMI 1640 was layered on lymphoprep and the mononuclear cells were isolated after centrifugation. The cells were washed twice in RPMI 1640 and transferred to Widal tubes at a concentration of 1–2 × 10^6 cells per 1 ml The cells were then incubated with 1 ml of a 1 per cent latex (Difco particle size 0 8 µm) suspension in medium for 30 min at 37 C The cells were spun down, the supernatant was discarded, and the following steps were carried out at 0 C Three drops of a 1 7 dilution of fluorescein-conjugated (FIT) polyvalent rabbit-antihuman Ig (DaKo, Copenhagen) were added and mixed with the cell pellet and allowed to conjugate for 30 minutes. Washing twice with 5 per cent albumin in RPMI 1640 followed and finally counting of wet sides in a Leitz fluorescence microscope at a magnification of 400 × One hundred non phagocytozing cells were counted and the percentage of cells with membrane-fluorescence was determined.

Test Protocol

After the diagnosis of renal carcinoma had been established by arteriography the patients were admitted to the trial Pre-operatively three to eight tests were first performed in each patient with the LMCT employing extracts from 2–6 allogeneic hypernephromas and corresponding normal renal tissue. In each test, at least two tumours and two extracts of normal renal tissue were used at concentrations of 10 50 100 300 400, and 600 µg of protein per ml. Significant differences in the capacity of different renal carcinoma extracts to induce leucocyte migration inhibition has earlier been shown (18) To avoid this variation, only tumour extracts of a well-defined antigenic strength (MIS of more than 20 per cent) were used (18) Any significant depression of the response in the LMCT was not found in the immediate postoperative period.

After completion of the series, a titration curve applying to each patient was drawn, showing LMCT reactivity at the above mentioned concentrations of tumour extract. These are shown in Figs. 1 and 2. The normal range in the LMCT was defined as the mean ± 2 SD of the MI's in the control groups (a–d) using the maximal, non-toxic concentration of tumour extract which is 400 µg of protein per ml This range was from MI = 0 79–1 19 after three leucocyte washings (14) The use of normal ranges has earlier been discussed in detail (14 15) In the statistical evaluation a numerical grading of TCMH in each patient was based on the lowest tumour extract concentration giving significant leucocyte migration inhibition or stimulation. Detailed description of TCMH grading has earlier been published (16, 17) The gradings used were as follows

- Grade 0 MI>0 79 MI<1 19 at all tissue extract concentrations employed except 600 µg per ml which has been shown to exert a significant inhibitory effect on leucocytes from controls (14 15)
- Grade 1 MI<0 79 MI>1 19 at 400 µg per ml, but not at lower concentrations
- Grade 2 MI<0 79 MI>1 19 at 400 and 300 µg per ml but not at lower concentrations.
- Grade 3 MI<0 79 MI>1 19 at 400 300 and 100 µg per ml but not at lower concentrations.
- Grade 4 MI<0 79 MI>1 19 at 400 300 100 50 or 10 µg per ml

The results obtained by grading of each patient in the series are shown in Table 1

With a view to GIC, two tests, one pre-operative and one 8 days after operation, were performed

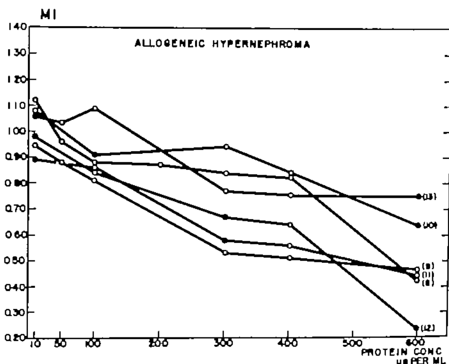


Fig 2 Titration curves applying to 6 patients with renal carcinoma indicating the presence of TCMH against allogeneic hypernephroma tissue extracts in the LMCT Numbers in parentheses refer to Table 1

patients, on the whole, differed from the control series in the transformation with PPD ($p<0.02$) and in membrane immunofluorescence ($p<0.05$) Otherwise the comparisons showed no significant differences. In 7 out of 13 patients responses to all antigens and mitogens were normal and besides, they presented a normal percentage of T rosettes. Three out of 6 patients with abnormal GIC had one abnormal response 2 out of six had two abnormal responses, and one out of 6 had more than two abnormal responses. As regards further statistical evaluation GIC was considered abnormal if only one response differed from that of control patients The scoring for each patient is shown in Table 1

No correlations between tumour pathology (Table 1) and TCMH or GIC were found

The correlations between TCMH GIC, and the clinical stage of the patients are shown in Tables 2–5 Certain trends point towards a positive correlation between the presence of distant metastases, a missing TCMH and an abnormal GIC (Table 2 and 3) but this was not significant in the present small series. Any correlation between the presence of TCMH and GIC was not found (Table 4) If however the correlation between the presence of distant metastases and a missing TCMH and/or an abnormal GIC were considered the correlation was significant (Table 1 Table 5)

DISCUSSION

The present investigation indicates that GIC is defective in about 50 per cent of patients with renal carcinoma in a consecutive series. It was not possible however to correlate the defects in GIC measured in the present study to the clinical stage of the lesion or to the histopathology of tumours.

Systematic studies of GIC in patients with renal carcinoma have been few *Brosman et al* (3) examined the monocyte function, the intracutaneous reactivity to recall antigens, and the reactivity to DNCB in 31 patients and found a progressive decline in reactivity to all antigens and mitogens with advancing

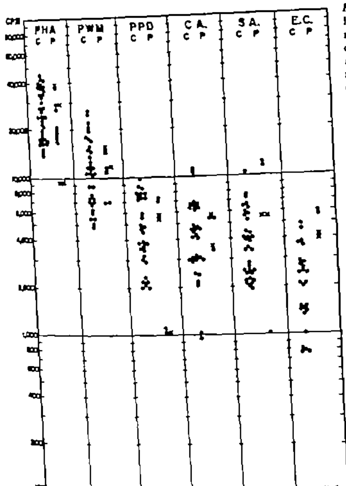


Fig. 3. Transformation values of lymphocytes from 13 patients with renal carcinoma (P) and from 37 controls (C) using phytohaemagglutination (PHA), pokeweed mitogen (PWM), purified protein derivative (PPD), *Candida albicans* (CA), *Staphylococcus aureus* (SA) and *Escherichia coli* (EC). Black circles indicate patients without distant metastases, crosses denoting patients with distant metastases.

tumour burden. Using transformation techniques, a decreased response to PHA has been observed in a few patients (1, 6, 23, 24), but stage-related reactivity was not a consistent finding (6). The main purpose of the present investigation was to study the possible correlation between the absence of TCMH against allogeneic hypernephroma tissue extracts and defects in GIC. Such correlation could not be demonstrated. To some extent this was surprising. A stage-related TCMH in patients with renal carcinoma has earlier been described by *Kjaer* (13–16). It would be natural to expect that absence of TCMH with advancing tumour burden might be explained at least partly by defects in GIC. However, our results indicate that, although there is a trend towards correlation between the presence of TCMH and intact GIC (patient no. 4, 5, 7, 9 and 13, Table 1) or the absence of TCMH and defect GIC (patients no. 3, 8, and 10, Table 1)

- defects in GIC are found in the presence of TCMH (patients nos. 2, 11 and 12, Table 1)
- intact GIC in patients without TCMH patients nos. 1 and 6, Table 1)

Thus defects in GIC could not explain the absence of TCMH in patients with renal carcinoma and distant metastases; however it is important to stress that in a greater

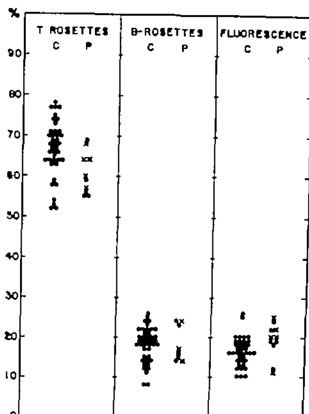


Fig 4 Percentage of T rosettes, B-rosettes, and direct membrane imunofluorescence of peripheral lymphocytes from 13 patients with renal carcinoma (*P*) and from 37 controls (*C*) *Black circles indicate patients without distant metastases, crosses* denoting patients with distant metastases.

number of patients a possible correlation between TCMH and GIC might become evident. The *finding of a significant correlation* to tumour stage if absence of TCMH and/or abnormal GIC was compared with intact responses in both TCMH and GIC indicates that both types of parameters may be of importance as a cause of immunological nonreactivity in patients with disseminated disease.

To our knowledge only one comparable study has been published (22) In patients with colonic cancer TCMH was tested by way of inhibition of mononuclear cell migration (IMM) and GIC by intracutaneous reactivity to recall antigens. In pre-operative tests using IMM all patients showed reactivity without stage-correlation but 10 out of 11 patients failed to react to two or more of the recall antigens employed. As regards the lack of correlation between TCMH and GIC the data obtained in the study by *Lurie et al* (22) and in the present study are in agreement. In most studies, however conclusions have been drawn on the basis of *defects in GIC to defects in* the immunological elimination of tumour cells *in vivo* (2 4 5 7 8, 9 23 24 27 31 33) Before

TABLE 1 *General Immunocompetence (GIC) and Tumour Directed Cell-Mediated Hypersensitiv* *Immunolog*

Patient No.	Clinical data: Sex	Age	Type of tumour	Metastases	Tumour pathology: Diameter of tumour cm	Macroscopic invasion	Microscopic invasion	Degree of differentiation
1	F	61	H	+	9	+	+	II
2	F	80	H	—	7	+	+	I
3	M	80	H	+	11	+	+	II
4	F	58	H	—	6	+	+	II
5	M	53	H		8	—	+	II
6	M	59	H	+	7	+	+	II
7	M	53	H	—	5	+	+	II
8	M	79	H	+	n	n.i	n.i	n.i
9	F	59	H		3	+	—	I
10	F	46	H	—	7	+	+	II
11	M	58	T	+	4	n.i.	+	n.i.
12	M	64	H	+	6	+	+	II
13	F	53	H	—	4	—	+	I

n.i. = not investigated. N = Normal. A = Abnormal H = Hypernephroma. T = Transitional ce

TABLE 2. *Correlation between TCMH and Clinical Stage*

	TCMH grade		
	0	1–4	
+ Distant metastases	4	2	p = 0.086
— Distant metastases	1	6	

TABLE 3. *Corr lation between GIC and Clinical St g*

	GIC		
	Abnormal	Normal	
+ Distant metastases	4	2	p = 0.21
— Distant metastases	2	5	

this can be done, two basis points must be elucidated,

1. Is there a significant correlation between GIC and TCMH
2. Is there a significant correlation between the presence of TCMH or an intact GIC and the prognosis of the patients?

So far no studies have been concerned with a systematic evaluation of these most important points. In a prospective study of a consecutive series of patients with renal carcinoma, however *Kjaer* (19) has recently demonstrated a positive, highly significant correlation between the presence of TCMH in the LMCT and the survival after operation. This suggests the presence of a rejection antigen on renal carcinomas *in vivo* detectable by LMCT *in vitro* In the present study any correlation between TCMH and CIC

CMH) in 13 Patients with Renal Carcinoma Clinical Data, Tumour Pathology and Summary f dings

TCMH TCMH grade	General immunocompetence							GIC	Abnormal GIC and/or 0 TCMH
	Lymphocyte transformation						Lymphocyte concentration/µl		
	PHA	PWM	PPD	CA	SA	T rosettes			
0	N	N	N	N	N	N	1,820	N	+
2	N	N	N	A	A	N	1 440	A	+
0	A	N	A	A	A	N	1,372	A	+
2	N	N	N	N	N	N	1,920	N	—
2	N	N	N	N	N	N	966	N	—
0	N	N	N	N	N	N	1,932	N	+
2	N	N	N	N	N	N	1,891	N	—
0	A	N	N	N	N	N	2,263	A	+
2	N	N	N	N	N	N		N	—
0	N	N	A	N	A	N	1,554	A	+
2	N	N	N	N	A	N	2,142	A	+
2	N	N	A	N	N	N	n	A	+
2	N	N	N	N	N	N	2,268	N	—

arcinoma of the renal pelvis.

27*

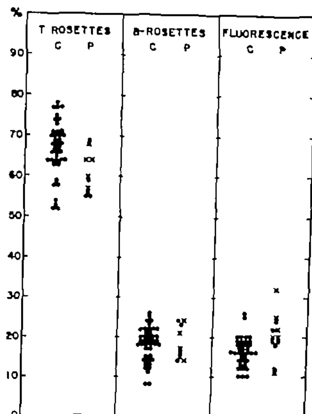


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5	M	53	H		8		+	II
6	M	59	H	+	7	+	+	II
7	M	53	H		5	+	+	II
8	M	79	H	+	n.i	n.i	n.i.	n.i.
9	F	59	H		3	+	−	I
10	F	46	H		7	+	+	II
11	M	58	T	+	4	n.i	+	n.i.
12	M	64	H	+	6	+	+	II
13	F	53	H		4		+	I

n.i. = not investigated N = Normal. A = Abnormal. H = Hypernephroma. T = Transitional ce

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+ Distant metastases	4	2	p = 0.066
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TABLE 3. *Correlation between GIC and Clinical Stage*

	GIC		
	Abnormal	Normal	
+ Distant metastases	4	2	p = 0.21
— Distant metastases	2	5	

this can be done, two basic points must be elucidated,

1. Is there a significant correlation between GIC and TCMH
2. Is there a significant correlation between the presence of TCMH or an intact GIC and the prognosis of the patients

So far no studies have been concerned with a systematic evaluation of these most important points. In a prospective study of a consecutive series of patients with renal carcinoma, however *Kjaer* (19) has recently demonstrated a positive, highly significant correlation between the presence of TCMH in the LMCT and the survival after operation. This suggests the presence of a rejection *antigen on renal carcinomas in vivo detectable by LMCT in vitro*. In the present study any correlation between TCMH and GIC

CMH) in 13 *Patients with Renal Carcinoma. Clinical Data Tumor Pathology and Summary of* ...ings

TCMH grade	PHA	PWM	PPD	CA	SA	T rosettes	Lymphocyte concentration/µl	GIC	Abnormal GIC and/or 0 TCMH
	General immunocompetence								
	Lymphocyte transformation								
0	N	N	N	N	N	N	1,620	N	+
2	N	N	N	A	A	N	1 440	A	+
0	A	N	A	A	A	N	1,372	A	+
2	N	N	N	N	N	N	1,920	N	—
2	N	N	N	N	N	N	966	N	—
0	Y	N	N	N	N	N	1 932	N	+
2	N	N	N	N	N	N	1,891	N	—
0	A	N	N	N	N	N	2,263	A	+
2	N	N	N	N	N	N	n.t	N	—
0	N	N	A	N	A	N	1,534	A	+
2	N	N	N	N	A	N	2,142	A	+
2	N	N	A	N	N	N	n	A	+
2	N	N	N	N	N	N	2,268	N	—

...noma of the renal pelvis.

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TABLE 4 *Correlation between TCMH and GIC*

	TCMH 0	TCMH 1–4	
Abnormal GIC	3	3	
Normal GIC	2	5	p = 0 41

TABLE 5 *Correlation between Clinical Stage and Abnormal GIC and/or TCMH Grade 0*

	TCMH 1–4 + normal GIC	TCMH 0 and/or abnormal GIC	
+ Distant metastases	0	6	
— Distant metastases	5	2	p = 0 016

could not be demonstrated but using both parameters a positive stage-related correlations was found. The lack of correlation between TCMH and GIC might also be explained by the existence of blocking antigen antibody complexes (18) circulating antigen (17) or other humoral factors operating in TCMH but not in GIC

In conclusion it will be highly difficult on the basis of investigations of GIC to conclude anything concerning TCMH and prognosis If investigations of cancer patients are to be of prognostic value it is mandatory that studies of TCMH as well as of GIC in prospective series of patients are as multifaceted as possible.

The study was supported by *The Danish Cancer Society* and by *The Daell Foundation* The authors want to thank Mrs. *Anne Kjerulff* Mrs. *Jette Hasting* and Mrs. *Lillian Merck* for competent technical assistance.

REFERENCES

1 *Al-Sarraf M Sardesai S & Vaitkevicius V K* Clinical immunologic responsiveness in malignant disease. II In vitro lymphocyte response to phytohemagglutinin and the effect of cytotoxic drugs. Oncology 26 357–368, 1972

2. *Anthony H M Kirk J A Madsen K E Mason M K & Templeman G H* E and EAC rosetting lymphocytes in patients with carcinoma of the bronchus. I Some parameters of the test and of its prognostic significance. Clin. exp. Immunol. 20 29–40, 1975.

3 *Brosman S., Hausman M & Sacks S J.* Studies on the immune status of patients with renal adenocarcinoma. J Urol. 114 375–380 1975

4 *Buttersworth C Oon C J Westbury G & Hobbs J R* T lymphocyte responses in patients with malignant melanoma. Europ. J Cancer 10 639–646, 1974

5 *Catalona W J Sample W F & Chretien P B* Lymphocyte reactivity in cancer patients Correlation with tumor histology and clinical stage. Cancer 31 65–71 1973

6 *Catalona W J Tarpley J L Chretien P B & Castle J R* Lymphocyte stimulation in urologic cancer patients. J Urol. 112 373–377 1974

7 *Catalona W J Potvin C & Chretien P B.* T lymphocytes in bladder and prostatic cancer patients. J Urol 112 378–382 1974

8 *Chretien P B Crowder W L Gertner H P Sample F & Catalona W J* Correlation of preoperative lymphocyte reactivity with clinical course of cancer patients. Surg Gynecol. Obst 136 380–384 1973

9 *Dellon A L Potvin C & Chretien P B.* Thymus-dependent lymphocyte levels in bronchogenic carcinoma Correlations with histology clinical stage, and clinical course after surgical treatment. Cancer 35 687 694 1975

10 *Golub S H O'Connell T X & Morton, D L.* Correlation of in vivo and in vitro assays of immunocompetence in cancer patients. Cancer Res. 34 1833–1837 1974

11 *Gross R L. Latty A Williams E. A & Newberne P M* Abnormal spontaneous ro-

ette formation and rosette inhibition in lung carcinoma. New Engl. J Med. 292 439–443 1975.

12. Kjaer M The capillary tube leucocyte migration technique (LMT) as a method for detection of serum inhibiting activity of tumour-directed, cellular hypersensitivity in patients with renal carcinoma. *Acta path. microbiol. scand.* Sect. B, 82 294–296 1974

13. Kjaer M In vitro demonstration of cellular hypersensitivity to tumour antigens by means of the leucocyte migration technique in patients with renal carcinoma. Europ J Cancer 10 523–531 1974

14. Kjaer M The dose-related effect of tumour extract on the in vitro migration of leucocytes from patients with renal carcinoma. Europ. J Cancer 11 281 289 1975

15. Kjaer M & Bendixen G Tumour-directed, cellular hypersensitivity detected by means of leucocyte migration technique in patients with renal carcinoma Ann. N Y Acad. Sci. 1976 (In press)

16. Kjaer M & Bendixen G Clinical detection and monitoring of tumour-specific cell mediated hypersensitivity (TCMH) in man Examinations of hypernephroma with leucocyte migration technique I *Bloom, B.* and *David, J R. (Eds.)* In vitro methods in cell-mediated immunity 1976 (In press)

17. Kjaer M Effect of leucocyte washings on cellular immunity to human renal carcinoma Europ. J Cancer 1976 (In press)

18. Kjaer M & Christensen, N Ability of renal carcinoma tissue extracts to induce leucocyte migration inhibition in patients with non-metastatic renal carcinoma Correlation with clinical and histopathological findings Cancer Immunol Immunother 1976 (In press)

19. Kjaer M Prognostic value of tumor-directed cell-mediated hypersensitivity detected by means of the leucocyte migration technique in patients with renal carcinoma Europ J Cancer 1976 (In press)

20. Knight L A & Davidson W M Reduced lymphocyte transformation in early breast cancer J clin Path. 28 372 376, 1975

21. Lehner T Shillitoe E J Wilton J M A & Ivanyi, L Cell mediated immunity to herpesvirus type 1 in carcinoma and pre-cancerous lesions. Br J Cancer 28 suppl. 1 128–134 1973

22. *Laurie B. B., Ball D M Zamcheck N., Steward A M & Hines R. A* Diagnosis and prognosis in colon cancer based on a profile of immune reactivity J Natl. Cancer Inst. 54 319–325 1975.

23. *McLaughlin A P Kressler W O Triman K & Gittes R. F* Immunologic competence in patients with urologic cancer J Urol. 111 233–237 1974

24. *McLaughlin, A P & Brooks J D* A plasma factor inhibiting lymphocyte reactivity in urologic cancer patients. J Urol. 112 366–372, 1974

25. *Nelson H S* Delayed hypersensitivity in cancer patients Cutaneous and in vitro lymphocyte response to specific antigens. J Nat Cancer Inst. 42 765–770 1969

26. *Platz P Fog T Morling, N Svejgaard A Sondergaard G Ryder L. P Thomsen M & Jersild C* Immunological in vitro parameters in patients with multiple sclerosis and normal individuals Acta path. microbiol. scand Sect C, 1976 in press.

27. *Potvin, C., Tarpley J L & Chretien, P B.* Thymus-derived lymphocytes in patients with solid malignancies. Clin. Immunol Immunopathol. 3 476–481 1975.

28. *Riesco A* Five-year cancer cure Relation to total amount of peripheral lymphocytes and neutrophils. Cancer 25 135–140 1970

29. *Sutherland R M Inch, W R & McCredie J A* Phytohemagglutinin (PHA)-induced transformation of lymphocytes from patients with cancer Cancer 27 574–577 1970

30. *Takeuchi S & Kanazawa, K* Changes of immunologic competence of lymphocytes evaluated by phytohemagglutinin (PHA)-induced DNA synthesis related to pregnancy and gynecologic tumors Acta Obst et Gynaec. Jap. 19 59–63 1972.

31. *Thomas P L & Chretien P B.* Impaired lymphocyte responsiveness in osteosarcoma. J Surg Res 18 551 554 1975

32. *Wells R M Burger D R & Lilley D P* Evaluation of immune status in tumor patients. Arch. Surg 108 558–560, 1974

33. *Whittaker M G & Clark C G* Depressed lymphocyte function in carcinoma of the breast. Brit. J Surg. 58 717 720 1971

Acta path. microbiol scand. Sect. C, 84 414-418 1976

THE EFFECT OF NITROGEN MUSTARD ON HUMAN MONONUCLEAR BLOOD CELLS

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Ødegaard, A. & Lamvik, J The effect of nitrogen mustard on human mononuclear blood cells. Acta path. microbiol scand. Sect. C, 84 414-418 1976

The effect of nitrogen mustard on the function of human blood monocytes and lymphocytes cultured *in vitro* was studied. After a single *in vitro* exposure to the drug at culture start, an inhibition of the survival of non proliferating mononuclear phagocytes was observed. No immediate cytotoxic effect was registered Nitrogen mustard given in therapeutic doses to patients with malignant lymphogranulomatosis did not reduce the survival of mononuclear phagocytes cultured at different times after the administration of the drug.

Key words Mononuclear blood cells nitrogen mustard.

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It is known from clinical observations and animal experiments that the host defence against microorganisms is reduced during treatment with cytostatic drugs. This may be partially due to an effect of these drugs on the mononuclear phagocytes. It has been shown that some cytostatic drugs have a direct effect on the proliferation of macrophage precursors (4) It is conceivable too that the same drugs, by an indirect effect on cell metabolism, can affect the functional capacity of the cells e.g differentiation and phagocytosis The aim of the present work was to study the direct effect of nitrogen mustard an alkylating agent, on mononuclear blood cells cultured *in vitro* and the *in vivo* effect of therapeutic doses of the same drug using standardized techniques for functional registrations.

MATERIALS AND METHODS

The general methods for culturing of human monocytes and for registration of drug effect on different functions of mononuclear blood cells have been reported in detail previously (7) Registration of cellular adhesiveness, preparation of rosette-forming lymphocytes and registration of survival of mononuclear adhesive cells after exposure to the drug were performed as described in test model I (7)

Monocytes cultured on coverslips were tested for phagocytosis after 8 days culturing The effect of nitrogen mustard on engulfment and on digestion of radiolabelled heat-killed Candida particles was studied in separate experiments and registered according to methods described previously as test model II (7) Nitrogen mustard (Mustine, Boots) was dissolved in and diluted with sterile distilled water

In the engulfment experiments, the cells were incubated with a constant number of Candida particles and nitrogen mustard in varying concentrations. The cells were harvested after a period of phagocytosis of 10 minutes The ratio between the number of Candida particles added and the number of adhesive phagocytes was usually about 0 1

TABLE 1 *Effect of Nitrogen Mustard on S r ral / Blood M on cle Phagocyte Cultured in* Vitro

Culture series no.	Numbers of surviving cells after exposure to nitrogen mustard in the following contrations, µg/ml					
	0	0.6	1.25	2.5	5	10
53	70	44	0	0	0	0
54	97	83	0	0	0	0
60	179	111	47	0	0	0
61	133	76	20	0	0	0
64	60	28	0	0	0	0
71	53	–	5	–	–	–

The cells were exposed to the drug for 90 min t culture start and the numbers of adheu e phagocytes counted after 4 days culturing. The alues given are the means of the cell numbers $\times 10^3$ in triplicate culture dishes.
not tested

In the digestion experiments, a constant number of Candida was added to 8 days old cell cultures. After a further incubation for 10 minutes, the coverslips were washed 3 times and placed in new petri dishes containing fresh culture medium. Nitrogen mustard in arying concentrations was added to the culture medium one hour later. The cultures ere harvested after incubation for fur ther 23 hours. The radioactivity on the coverslips was registered as measure of the amount of non-digested Candida left in the cells on the coverslips. The radioactivity in the cell free medium and in the sediment following high speed centrifugation was measured separately.

The radioactivity in the call free medium is a measure of the digestiv capacity of the phagocytes, hile the radioactivity in the sediment after high speed centrifugation is measure of the number of detached cells containing non-digested Candida in addition to small number of non-phagocytized Candida particles.

Mononuclear cells from three patients with lymphogranulomatosis were separated from enous blood before, as well as 90 minutes and 8 day after single intravenous injection of 10 mg nitrogen mustard. The cells were cultured and the functional capacities were registered according to the procedures described abov.

The patients were all males of ages between 30 and 60 years in whom the disease was in stage IV. I one of the patients (L.S.) the test was performed before any other cytostatic drugs had been given, at time when he was acutely ill with fever malaise and general lymph node enlargement. I another patient (K.R.) the test was done one month following two-week-course of treatment with nitrogen mustard, ablastin, procarbazine and prednisone. I the third patient (J.S.) the culture experiments were performed one month after the last of f similar courses of drug therapy. The two last patients were both symptom-free at the time of the test.

RESULTS

In the *in vitro* experiments, the mononuclear blood cells were incubated with nitrogen mustard for a period of 90 minutes at culture start. After this period of time, the cells which had been exposed to the drug showed no morphological alterations or alterations of functional properties such as ability to attach to glass or plastic surfaces or to engulf par

TABLE 2 *Eff ct of Nitrogen Mustard on R sette-forming Ability of Lymphocytes and on Engulfment Capacity / Mononuclear Phagocytes*

Drug conc. µg/ml	Numbers of rosette-forming lymphocytes	Engulfment capacity§
0	25.8 (± 3.7)	288 (± 41)
0.6	20.6 (± 4.6)	287 (± 25)
1.25	20.4 (± 4.7)	289 (± 62)
2.5	19.9 (± 5.8)	295 (± 63)
5	20.1 (± 8.5)	301 (± 57)
10	20.0 (± 6.3)	286 (± 44)

The figures listed are the means ± SD of the results from 6 experiments.
The numbers are given as percentages of the total lymphocyte count.
§ Radioactivity ct/min $\times 10^3$ per culture dish.

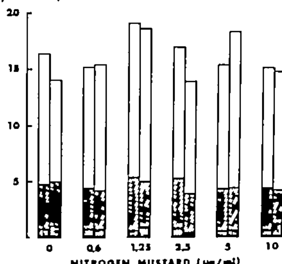


Fig 1 Effect of different concentrations of nitrogen mustard on digestion of engulfed ^{125}I-labelled heat killed Candida particles in 8 days old blood mononuclear phagocytes cultured *in vitro* The figures are the results obtained in one characteristic digestion experiment performed in duplicate culture dishes. Radioactivities in different parts of the culture system are given.

ticles. A single exposure of nitrogen mustard in final concentrations ranging between 0 6 µg/ml and 10 µg/ml at culture start inhibited the survival of adhesive mononuclear phagocytes, registered after four days of culturing (Table 1)

Following 90 minutes of exposure to nitrogen mustard the ability of lymphocytes to form rosettes with sheep red blood cells was slightly reduced (Table 2)

No direct effect of nitrogen mustard on the engulfment of Candida particles was registered and the same applies to the intracellular digestion of engulfed particles in 8 days old mononuclear phagocytes (Table 2 Fig 1 and 2)

A single i v injection of 10 mg nitrogen mustard to the patients with malignant lymphogranulomatosis did not result in a functional reduction in lymphocytes or monocytes separated and cultured 90 minutes and 7 days after administration of the drug (Table 3) The functional abilities of the cells were found to be similar to those in cells from normal individuals.

DISCUSSION

In the present study nitrogen mustard was found to inhibit the survival of adhesive mononuclear phagocytes *in vitro* registered on the fourth day of culturing after a single exposure to the drug at culture start. No immediate cytotoxic effect, leading to impaired cytoplasmic functions, was seen to follow exposure to the drug

Nitrogen mustard interacts with cells causing interstrand binding in the DNA molecule, leading to prevention of DNA replication (3) It appears that its primary effect on the cell nucleus occurs within a few seconds. The mechanism by which alkylating agents inhibit the immune response are not fully understood but *Santos & Owens* found that these drugs were not effective in suppressing immune response except if they were given before exposure to antigen (5) No single satisfactory *in vitro* test for the evaluation of im-

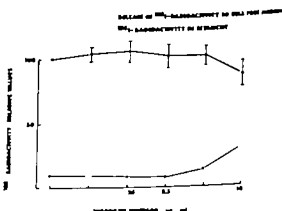


Fig 2 Effect of nitrogen mustard on digestion of ^{125}I labelled heat killed Candida particles in blood mononuclear phagocytes cultured for 8 days on coverslips. The values are the means of ± SD of the results obtained in 6 experiments performed in duplicate culture dishes and registered 23 hours following the engulfment phase. Results are expressed as percentages of the radioactivity in the medium in control cultures without drug addition.

TABLE 3. *The Effect of a Single I.v. Injection of 10 mg Nitrogen Mustard on Cultured Mononuclear Blood Cells from Three Patients with Malignant Lymphogranulomatosis*

Patient		Rosette-forming ability of lymphocytes (per cent of lymphocyte count)	Cellular adhesiveness (cell number per culture dish × 10³)	Survival (cell number per culture dish × 10³)	Engulfment capacity (radioactivity ct/min × 10³ per culture dish)	Digestion capacity (per cent of total radioactivity found in cell free medium)
L.S.	I	14.4	720	46	90	65
	II	12.1	1020	52	89	66
	III	16.0	1500	78	220	71
K.R.	I	30.7	810	66	225	63
	II	32.0	810	68	209	60
	III	24.5	840	97	162	68
J.S.	I	22.3	930	87	220	71
	II	25.8	1080	88	223	68
	III	24.2	830	75	215	76

I = cell culture prepared before administration of drug.
II = cell culture prepared 90 minutes after administration of drug.
III = cell culture prepared 7 days after administration of drug.
The figures listed are the means of the results from duplicate (rosettes, adhesiveness, survival) or triplicate (engulfment, digestion) culture dishes.

immunosuppressive drug effect in man has been developed. However it has been found that there is good correlation between the suppressive effect on the ability of lymphocytes to form rosettes with sheep red blood cells and the immunosuppressive potency of antilymphocyte sera (1). In previously reported experiments (8) azathioprine, an antimetabolite, was found to inhibit the rosette forming ability of human lymphocytes in concentrations below the level of cytotoxicity. A comparison between non-toxic concentrations of azathioprine and nitrogen mustard indicates that azathioprine is a better immunosuppressant if the effect on the rosette forming ability of lymphocytes is taken as a sign of immunosuppression.

Nitrogen mustard was not found to affect the blood clearance of carbon particles in mice (2). However, when nitrogen mustard was given to mice that had been saturated with carbon, recovery of the ability to clear carbon particles was found to be delayed (2) which may be explained by an inhibitory effect of the drug on the proliferation of macrophage precursors. Inhibition of phagocytosis may thus be linked firstly to a reduced number of cells capable of phagocytosis, either due to decreased proliferation of phagocytic precursors or to a lack of differention of precursor cells, or secondly to reduced phagocytic ability of single cells. The results presented indicate that the phagocytic ability of macrophages is not directly affected by nitrogen mustard even in concentrations close to the level of cytotoxicity. Indirectly however this important cell function may be impaired by the effect of nitrogen mustard on the precursor cells of macrophages, as indicated in this study by the impaired survival of monocytes *in vitro*.

When nitrogen mustard was given in therapeutic concentrations to patients with malignant lymphogranulomatosis, the numbers of adhesive monocytes were not affected. This observation is in accordance with the finding by *Ziff et al.* (6) who observed that cyclophosphamide, an alkylating agent, did not reduce the blood monocyte count in mice. In contrast to the results of the *in vitro* experi-

ment where a single exposure to nitrogen mustard inhibited the survival of monocytes no corresponding effect on monocytes exposed to nitrogen mustard *in vivo* was found This finding may be due to the fact that the concentration of nitrogen mustard obtained after a single injection of 10 mg nitrogen mustard was lower than that used in the *in vitro* experiment Another explanation of the apparent lack of inhibitory effect of nitrogen mustard on monocyte function after intravenous administration may be related to the duration of the drug effect on the monocytes compared with the turnover time of the cells. If the latter is very short, a high number of monocytes without previous contact with nitrogen mustard may be obtained from the blood 90 min after drug administration The results of the purely *in vitro* experiments as well as those of the therapeutic trials indicate that alkylating agents cause little harm to mononuclear phagocytic functions.

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REFERENCES

1 *Bach J F Dardenne M & Fournier C.* In vitro evaluation of immunosuppressive drugs. Nature 222 998–999 1969

2 *Benacerraf B Halpern B. N Biozzi G. & Benos S A* Quantitative study of the granulopectic activity of the reticulo-endothelial system III The effect of cortisone and nitrogen mustard on the regeneration capacity of the R.E.S after saturation with carbon. Brit. J Exp Pathol. *35* 97–106 1954

3 *Crathorn A R & Roberts J J* Mechanism of the cytotoxic action of alkylating agents in mammalian cells and evidence for the removal of alkylated groups from desoxyribonuclei acid. Nature *211* 150–153 1966.

4 *van Furth R Gassmann A E., Diesselhoff Den Dulk M* The effect of azathioprine (IMURAN®) on the cell cycle of promonocytes and the production of monocytes in the bone marrow J Exp Med. *141* 531–546 1975

5 *Santos G W & Owens A H Jr* The effect of certain alkylating agents and antimetabolites on the primary agglutinin response of rats injected with sheep erythrocytes. Blood *20* 111 1962

6 *Ziff M Hurd E R Lemmel E. M & Jasin H E* The effect of cytostatic agents on the kinetics of mononuclear phagocytes. In *van Furth R* (Ed.) Mononuclear Phagocytes, 1 ed Blackwell Scientific Publications, Oxford 1970 p 282–298.

7 *Ødegaard A* Functional alterations induced by chlorpromazine in mononuclear blood cells cultured in vitro Acta path. microbiol. scand. Sect. C *83* 59–73 1975

8. *Ødegaard A* The effect of azathioprine on mononuclear blood cells cultured in vitro. Acta path. microbiol. scand. Sect C, *83* 74 82 1975

Acta path. microbiol. scand. Sect. C, 84 419–424 1976

EFFECT OF AUROTHIOMALATE ON HUMAN MONONUCLEAR BLOOD CELLS CULTURED *IN VITRO*

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Human mononuclear phagocytes were exposed to aurothiomalate in various concentrations and at various stages in the differentiation from monocytes to macrophages *in vitro* Monocytes exposed to aurothiomalate during the first 90 minutes of culture showed impaired engulfment capacity when tested 8 days after the exposure to the drug. It was found that aurothiomalate suppressed the digestion capacity in differentiated macrophages while the engulfment capacity was unaffected by the drug. During the period of differentiation from 90 minutes to 8 days of culture, exposure to aurothiomalate resulted in a dose dependent reduction in cell survival and differentiation. The effect of aurothiomalate on the blastoid transformation of lymphocytes following BCG stimulation was also tested. A strong and dose dependent inhibition of DNA synthesis was recorded. The inhibition of phagocytosis in mononuclear phagocytes and the inhibition of antigen-induced lymphocyte stimulation as demonstrated may help to explain the effect of aurothiomalate in patients suffering from rheumatoid arthritis.

Key words Mononuclear phagocytes lymphocytes effect of aurothiomalate *in vitro*

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The clinical effect of aurothiomalate in the treatment of rheumatoid arthritis is well documented (1) The effect of chrysotherapy occurs only after 2–5 months of treatment and may persist for 6–12 months after administration is discontinued (2) This indicates an accumulation of gold in the body during chrysotherapy Measurements of the retention of gold during therapy document the accumulation of gold in the tissues (2)

Our knowledge about the effect of aurothiomalate at the cellular level is still limited. It has been shown that gold inhibits lysosomal enzymes *in vitro* (8 9) It has also been demonstrated that gold salts suppress phagocytosis both *in vitro* and *in vivo* (5)

The mononuclear phagocyte and the lymphocyte are important cells in the chronic inflammatory reaction. The purpose of this investigation was to study the effect of aurothiomalate on these cells.

MATERIALS AND METHODS

Human mononuclear blood cells were separated from the blood of healthy volunteers as previously described (16) The general procedures for testing the cells have been reported in previous papers (12 13 14 17)

Aurothiomalate

Na aurothiomalate (Myocrisin® "Rhodia Denmark) was dissolved and diluted in 0 9 per cent NaCl before addition to the culture dishes.

^{125}I-labelled Candida albicans

Candida albicans (Dept. of Microbiology Trondheim Regional Hospital) heat killed at 60 C for 2 hours were labelled with ^{125}I (Kjeller Norway) by means of electrolysis (11)

Culture Media

The mononuclear cells, separated from human, defibrinated venous blood were usually cultured in medium R.P.M.I 1640 (Flow G.B.) supplemented with 25 per cent pooled human A serum, 10 µg/ml glutamin and 50 µg gentamicin per ml. In the lymphocyte-stimulation experiments (test procedure 4) medium containing R.P.M.I 1640 L-glutamin gentamicin and 20 per cent foetal bovine serum, Flow G.B.) was used.

Test Procedure 1

The mononuclear cell suspension was exposed to aurothiomalate during the first 90 minutes of culture. Adhesiveness and survival were measured as previously described (13 17) These tests were performed in plastic petri dishes (Nunclon, Denmark) The number of adhesive cells was calculated as being the difference between the number of cells added at culture start and number of non attached cells removed by washing in Hanks balanced salt solution (Hanks B.S.S.) The washing procedure was carried out after 90 minutes incubation at 37 C in a National CO_2 incubator containing 5 per cent CO_2 in air and with 100 per cent humidity The cell numbers were recorded in an electronic particle counter (Coulter type FN) The survival of the mononuclear adhesive cells was estimated by counting the numbers of cells remaining 8 days after the exposure to the drug using an inverted phase contrast microscope (Reichert Austria)

Mononuclear blood cells cultured on coverslips were also exposed to aurothiomalate during the first 90 minutes of culture This method allowed functional testing of the cells 8 days after this short exposure to the drug The culture medium was removed on the 8th day of culturing medium containing 2 × 10⁶ ^{125}I labelled *Candida albicans* per ml was added, and the culture dishes were incubated for 15 minutes at 37 C The 2 cover slips in each petri dish were then picked out, rinsed 12 times in culture medium and placed in a new petri dish. To each dish 2.5 ml culture medium was added. After culturing for 24 hours, the 2 coverslips were removed the medium harvested, centrifuged at 2000 *G* for 10 minutes and the sediment and supernatant were collected separately The two coverslips, the sediment and the cell free medium were then counted for radioactivity in an Wallac GM γ-scintillation counter The radioactivity left on the two coverslips represents intracellular undigested Candida and the radioactivity in the sediment represents undigested Candida left in detached cells or free Candida particles. The radioactivity found in the cell free medium represents digested yeast material, and the total radioactivity per culture dish represents the number of phagocytized Candida particles.

Test Procedure 2

Mononuclear blood cells were cultured on cover slips for 8 days with no drug addition (14)

a. *Engulfment capacity* After removal of the culture medium the cells were exposed to various concentrations of aurothiomalate together with 2 × 10⁶ radiolabelled Candida particles per ml culture medium. The cells were incubated at 37 C for 15 minutes and the coverslips were removed, washed 2 × 6 times in Hanks B.S.S. and the amount of radioactivity was registered

b *Digestion capacity* The general procedure for the digestion experiments has been reported in detail earlier (12, 14 17) Aurothiomalate was added to the culture dishes 1 hour after engulfment of radiolabelled *Candida albicans* performed as described in test procedure 1 The digestion capacity is calculated as the percentage of the total radioactivity found in the cell free portion of the medium.

The digestion experiments were also performed after half an hour's pre-incubation with the various concentrations of aurothiomalate. Further the drug was added during the engulfment period of 15 minutes and immediately after the coverslips had been transferred to the new culture dishes. In these experiments the total radioactivity per culture dish reflects the phagocytic capacity i.e. the amount of radiolabelled Candida attached or engulfed by the cells on the 2 coverslips that had been transferred to the new culture dish.

Test Procedure 3

In these experiments, the effect of the drug on the differentiation of monocytes to macrophages was studied The drug was added to the cells after medium changes at 90 minutes, 1 and 4 days. After culturing for 8 days, the cells on the cover slips were counted in an inverted phase contrast microscope (Reichert, Austria) The cells were then tested for phagocytic ability using ¹I-labelled *Candida albicans* as described in test procedure 1

Test Procedure 4

In these experiments, the effect of aurothiomalate on the blastoid transformation of lymphocytes after antigen-stimulation was tested. The antigen used was BCG-bacilli (Statens Seruminstitut, Denmark) suspended in culture medium at a final concentration of approximately 10^7 bacilli per ml. The donors used in these experiments showed a positive tuberculin reaction after vaccination.

The mononuclear cell suspension was adjusted to 1×10^6 cells per ml and added to culture tubes (Kimax) in a volume of 2.5 ml per culture tube. Aurothiomalate in various concentrations and BCG-bacilli were added. The culture tubes were centrifuged at 200 *G* for 10 minutes before incubation at 37° C with 5 per cent CO_2 in air and with 100 per cent humidity.

After culturing for 5 days, 0.5 μCi per ml of ^{3}H-thymidine was added to each tube, and the cells were harvested 18 hours later. The cells were centrifuged at 400 *G* for 10 minutes at 4° C and washed once in 0.9 per cent NaCl. 0.02 ml of serum (carrierprotein) and 2 ml of cold 5 per cent trichloracetic acid (TCA) were added per culture tube. After 24 hours at 4° C the sediments were washed once in 2 ml cold 5 per cent TCA, once in 2 ml cold methanol, dried at 37° C and then dissolved in 0.5 ml Hyamin (Rohm & Haas, USA) before addition of liquid scintillation fluid (4). The amount of radioactivity in the samples were counted in a liquid scintillation counter (Isocap/300, Nuclear Chicago, USA).

Statistics

The experiments were performed in duplicate or triplicate culture dishes or in 5-plicate culture tubes. In each experiment, the mean values from replicates containing the same drug concentration were calculated, and the results were expressed as percentage of control values without drug exposure. The figures presented are the mean values from 5–8 experiments ± standard deviation (SD). The p-values were calculated using the Wilcoxon Two-Sample test.

RESULTS

Cells exposed to aurothiomalate during the first 90 minutes of culture showed no alteration in their ability to adhere to plastic surface or to survive compared to control cell cultures (Table 1). The cells left 8 days after exposure to the drug, however, showed a decreased ability to engulf the yeast particles in doses exceeding 10 μg/ml ($p<0.01$). The *digestion of the engulfed particles* was not influenced by the exposure to aurothiomalate 8 days earlier ($p>0.05$) (Table 1).

When cells cultured for 8 days without drug addition were tested, no significant effect on the engulfment of yeast particles in the presence of aurothiomalate in doses up to 100 μg/ml could be recorded ($p>0.05$). After pre-incubation with aurothiomalate for half an hour prior to the commencement of the test, only a slight reduction of the engulfment capacity was found in cells exposed to a concentration of 100 μg/ml of the drug ($p<0.05$) (Table 2). The digestion capacity in differentiated macrophages was found to be reduced in the presence of aurothiomalate

TABLE 1. *The Effect of Aurothiomalate on Various Functions of Mononuclear Blood Cells Exposed During the First 90 Minutes of Culture*

Drug conc. μg/ml	Numbers of adherent cells	Survival	Engulfment capacity	Digestion capacity
100	108 ± 27	120 ± 56	65 ± 8	95 ± 5
10	105 ± 29	105 ± 27	70 ± 21	96 ± 4
0	100*	100*	100*	100*

The figures listed are the means ± SD of the results from 8 experiments carried out as described in test procedure 1. The results are expressed as per cent of control values. The experiments were performed in duplicate or triplicate petri dishes.

* Control without drug addition. Numbers of adherent cells: 2.28 ± 1.08, range 0.85–3.85 × 10^6 adherent cells per culture dish. Survival: 380 ± 270, range 118–808 × 10^3 cells per culture dish after 8 days of culturing. Engulfment capacity: 62 ± 19, *range* 32–85 × 10^3 ct/min, radioactivity per culture dish. Digestion capacity: 76 ± 5, range 69–82 per cent radioactivity found in the medium.

TABLE 2 *The Effect of Aurothiomalate on Phagocytic Capacity of Human Macrophages Cultured for 8 Days before Testing*

Drug conc. µg/ml	Engulfment capacity		Digestion capacity	
	A	B	A	B
100	81 ± 16	98 ± 9	68 ± 6	65 ± 8
50	96 ± 10	100 ± 11	80 ± 9	76 ± 8
10	89 ± 14	98 ± 8	89 ± 8	89 ± 8
5	94 ± 17	99 ± 5	93 ± 6	94 ± 7
0	100	100*	100*	100

A Pre-incubated with drug for half an hour before test start.
B No pre incubation. The figures listed are the means ± SD of the results from A 5 – B 10 experiments, carried out as described in test procedure 2 The results are expressed as per cent of control values. The experiments were performed in triplicate petri dishes.
* Control without drug addition Engulfment capacity A 100 ± 55 range 10–185 × 10^3 ct/min, radioactivity per culture dish. B 80 ± 15 range 66–97 × 10^3 ct/min, radioactivity per culture dish. Digestion capacity A 74 ± 3 range 72–76 per cent radioactivity found in the medium. B 69 ± 5 range 64–76 per cent radioactivity found in the medium.

(Table 2) Pre incubation with the drug for half an hour and drug exposure during the engulfment stage of phagocytosis did not increase this effect (Table 2)

The effect of aurothiomalate on the differentiation of monocytes to macrophages was found to be more prominent. Addition of the drug in concentrations of 50 and 100 µg/ml after the medium changes at 90 minutes, 1 day and 4 days resulted in an almost complete absence of cells on the coverslips on the 8th day of culturing (Table 3) The few cells left, however did engulf *Candida albicans* though the digestion of the engulfed particles was markedly decreased (Table 3) In concentrations of 5 and 10 µg/ml of aurothiomalate, the number of cells left on the coverslips were significantly decreased compared to the control ($p<0.01$) The number of engulfed par

TABLE 3 *The Effect of Aurothiomalate on the Differentiation of Monocytes to Macrophages During the First 8 Days in Culture*

Drug conc. µg/ml	Number of cells left after 8 days	Engulfment capacity	Digestion capacity
100	1 ± 1	4 ± 2	41 ± 12
50	1 ± 1	3 ± 1	53 ± 16
10	58 ± 33	45 ± 26	88 ± 9
5	71 ± 19	73 ± 8	97 ± 4
0*	100*	100*	100*

The figures listed are the means ± SD of the results from 5 experiments, carried out as described in test procedure 3 The results are expressed a per cent of control values. The experiments were performed in triplicate petri dishes
* Control without drug addition Number of cell left after 8 days 320 ± 230 range 58–529 × 10^3 cells per culture dish. Engulfment capacity 142 ± 54 range 48–185 × 10^3 ct/min radioactivity per culture dish. Digestion capacity 77 ± 5 range 71–85 per cent radioactivity found in the medium.

TABLE 4 *The Effect of Aurothiomalate on ³H Thymidin incorporation in Lymphocytes after BCG-Stimulation*

Drug conc µg/ml	Radioactivity ct/min × 10	% of control values
100	44 ± 18	11 ± 6
50	117 ± 45	27 ± 6
10	247 ± 90	57 ± 13
5	360 ± 120	62 ± 8
0	431 ± 120*	100

The figures listed are the means ± SD of the results from 6 experiments, carried out as described in test procedure 4 The experiments were performed in 5-plicate culture tubes.
* Range 261–575 × 10 ct/min.

ticles per coverslip was reduced proportionally indicating no alteration in the ability of single cells to engulf *Candida* particles. The digestion capacity of the cells left on the coverslips exposed to a concentration of 10 µg/ml was found to be decreased, possibly indicating an impaired differentiation of these cells ($p<0.05$) (Table 3). Microscopic examination of the cells after 8 days exposure to aurothiomalate in concentrations of 50 and 100 µg/ml revealed a few scattered poorly differentiated cells left on the coverslips. Cells treated with aurothiomalate in concentrations of 5 and 10 µg/ml showed a slight reduction in size and perinuclear granulation.

When mononuclear blood cells were stimulated with BCG in the presence of various concentrations of aurothiomalate, a dose dependent decrease in DNA-synthesis was found as measured between the 5th and the 6th day of culture by means of H-thymidine incorporation (Table 4).

DISCUSSION

Gold is accumulated in the tissues during chrysotherapy and this seems necessary for the clinical effect of the drug. It is likely to assume that the intracellular concentration of gold exceeds the plasma concentration of approximately 3 µg/ml during therapy (2, 10). Beside high content of gold in the excretory cells in the renal tubulus, gold accumulate in lymphoid tissues and to some extent in the liver, both tissues being rich in macrophages (3). The increased accumulation of gold in the inflamed synovial tissue (6) may indicate accumulation in cells participating in chronic inflammation. Further it has been shown that gold accumulate in the lysosomes of guinea pig peritoneal macrophages (9). Colloidal gold injected into inflamed joints accumulates in the lysosomes of macrophages in the synovial tissue (15).

One of the theories for the pathogenesis of rheumatoid arthritis is a leakage of lysosomal enzymes as cause of the inflammatory reaction. It has been shown that gold inhibits several lysosomal enzymes such as acid phosphatase, β-glucuronidase (9) and the cathepsin d-type proteinase (8). The direct inhibition of these enzymes may explain the decreased digestion of yeast particles in the presence of aurothiomalate as found in our experiments. This is supported by the following findings. First, an immediate effect of the drug on digestion in differentiated macrophages while the engulfment stage of phagocytosis was allowed to go unaffected by drug addition. Secondly pre-incubation with aurothiomalate and exposure to the drug during the engulfment period did not increase the inhibitory effect on the digestion capacity. Thirdly no direct inhibition of the membrane function could be detected, measured as the ability of the cells to engulf yeast particles. Thus it seems that the effect of aurothiomalate on differentiated macrophages is mainly an inhibition of their digestion capacity, probably by inhibition of lysosomal enzymes.

Another anti-inflammatory effect of aurothiomalate has been shown to be an inhibition of the engulfment stage of phagocytosis *in vitro* and *in vivo* (5) measured as reduced uptake of carbon in neutrophil granulocytes and in macrophages during chrysotherapy. Beside the inhibition of the digestion stage of phagocytosis mentioned above, a reduced engulfment capacity was observed in cells treated for a short time with aurothiomalate 8 days prior to the testing of phagocytosis. This may indicate that a short time exposure of monocytes to aurothiomalate in high concentrations may induce membrane alterations, making the macrophages less capable to engulf particular material. Since the digestive function of the cells treated in this way remained unaffected, aurothiomalate did not seem to induce damage to the lysosomal membranes.

Another finding in our study was the marked depression of the differentiation of monocytes to macrophages when the cells were exposed continuously to aurothiomalate from 90 minutes to 8 days in culture. This situation is comparable to the conditions *in vivo* during chrysotherapy. When the cells were cultured in the presence of the drug in

concentrations exceeding 10 $\mu g/ml$ a strong and dose dependent suppressing effect on the cell survival was recorded and the remaining cells were morphologically and functionally found to be less differentiated compared with control cell cultures. This finding may indicate a suppressed differentiation of monocytes to macrophages at the site of inflammation during chrysotherapy, possibly resulting in a reduced number of cells with high content of lysosomal enzymes, with reduced capability of destroying the tissue by release of these enzymes.

The ability of lymphocytes to respond to antigen with proliferation measured as increased DNA synthesis was found to be strongly inhibited when the cell cultures were exposed to aurothiomalate. Lymphocytes are important cells with regard to chronic inflammations, both as effector cells towards antigens and as mediator cells for macrophage activation by production of lymphokines (7). However since the existence of an antigen as the cause of rheumatoid arthritis is unknown, the clinical importance of this finding is uncertain.

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REFERENCES

1. *Empire Rheumatism Council*. Gold therapy in rheumatoid arthritis. Final report of a multi-centre controlled trial. Ann Rheum Dis *20* 315–334 1961
2. *Gerber R C Paulus H E Jennrich R I Lederer M Bluestone R Bladh W H & Pearson C M*. Gold kinetics following aurothiomalate therapy. Use of a whole-body radiation counter. J Lab. Clin. Med. *83* 778–789 1974
3. *Gottlieb N L Smith, P M & Smith F M*. Tissue and fluid gold concentrations in a rheumatoid arthritic receiving chrysotherapy (abstr.) Arthr Rheum. *14* 164 1971
4. *Huges D & Caspari E. A*. Lymphocyte transformation in vitro measured by tritiated thymidin uptake. I. Lymphocyte culture techniques. Int. Arch. Allergy *37* 506–531 1970.
5. *Jessop J D Vernon Roberts B. & Harris J*. Effects of gold salts and prednisolone on inflammatory cells. I. Phagocytic activity of macrophages and polymorphs in inflammatory exudates studies by a skin-window technique in rheumatoid and control patients. Ann. Rheum. Dis. *32* 294–300 1973
6. *Lawrence J S*. Studies with radioactive gold. Ann. Rheum. Dis. *20* 341–351 1961
7. *Mackaness G B*. The influence of immunologically committed lymphoid cells on macrophage activity *in vivo*. J Exp. Med. *129* 973–992 1969
8. *McAdoo M H Dannenber A M Hayes C J James S P & Sanner J H*. Inhibition of the cathepsin d type proteinase by pepstatin, a specific pepsin inhibitor and other substances. Inf and Imm. *7* 655–665 1973.
9. *Persellin R H & Ziff M*. The effect of gold salts on lysosomal enzymes of the peritoneal macrophage. Arth. Rheum. *9* 57–65 1966.
10. *Rubenstein H M & Dietz A. A*. Serum gold. II. Levels in rheumatoid arthritis. Ann. Rheum. Dis. *32* 128–132, 1973
11. *Viken K E*. ^{125}I-labelling of *Canaida albicans* by electrolysis. Acta path. microbiol. scand. Sect. B, *82* 219–2?2 1974
12. *Viken K E. & Ødegaard A*. Phagocytosis of heat killed radiolabelled *Candida albicans* by human blood monocytes cultured *in vitro*. Acta path. microbiol. scand. Sect. B *82* 235–244 1974
13. *Viken K E*. The effect of steroids on adhesiveness, rosette-forming ability and survival of cultured human mononuclear cells. Acta path. microbiol. scand. Sect. C *84* 5–12 1976
14. *Viken K E*. The effect of steroids on differentiation and function of cultured mononuclear cells. Acta path. microbiol. scand. Sect. C *84* 13–22 1976.
15. *Yarom R Hall T H Stein H., Robin G C & Makin M*. Identification and localization of intraarticular colloidal gold. Ultrastructural and electron microprobe examinations of human biopsies. Virchows Arch. Abt. B Zell path. *13* 11–22 1973
16. *Ødegaard A Viken K E & Lamvik J O*. Structural and functional properties of blood monocytes cultured *in vitro*. Acta path. microbiol. scand. Sect. B *82* 223–234 1974
17. *Ødegaard A*. Functional alterations induced by chlorpromazine in mononuclear blood cells cultured *in vitro*. Acta path. microbiol. scand. Sect. C *83* 59–73 1975

BRIEF REPORTS

C1r LEVELS IN NORMAL HUMAN SERA DETERMINED BY ELECTROIMMUNOASSAY

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Sjöholm, A. G Mårtensson, U & Laurell, A. B. C1r levels in normal human sera determined by electroimmunoassay Acta path. microbiol. scand. Sect. C, 84 425–427 1976.

C1r levels in sera from 100 normal adults were determined by electroimmunoassay The 95 per cent range was 71 133 per cent of normal reference pool. C1r values were well correlated to the levels of C1q (r = 0.708) and of C1 (= 0.768) The interplate variation of the method on double determinations was 3.4 (SD) C1r values in normal sera not appreciably affected by storage at room temperature or by repeated freezing and thawing. The C1r antigen in EDTA plasma was found to be labile.

Key words Complement C1 quantitation electroimmunoassay

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Quantitation of complement components by electroimmunoassay has been shown to be a precise and time-saving procedure, well suited for large-scale clinical applications (5, 6, 7 8, 13) Few reports concern the quantitative estimation of C1r in serum (1 2, 3, 4 12) In the present paper the electroimmunoassay was employed for determination of C1 in 100 normal sera The technical procedure was described The stability of the C1 antigen in serum and EDTA plasma on storage at room temperature and on repeated freezing and thawing was also investigated

Materials and Methods

Serum and EDTA plasma Serum and EDTA plasma from 100 registered blood donors were handled as described previously (13) A reference serum pool was prepared and stored at –80° C in small portions that were used only once for analysis.

Serum and EDTA plasma from three individuals were divided into aliquots and frozen at –80 C after 2 hours of sampling or after storage at room temperature for 1 3 and 5 days. Aliquots were also repeatedly frozen at –80° C and thawed t room temperature up to three times before analysis.

Rabbit antiserum to C1 C1r was prepared from neutral euglobulin by chromatography on DE 52 cellulose, Sephadex G 200 and preparative agarose electrophoresis as will be reported separately (9) Rabbits were immunized by single subcutaneous dose of C1 emulsified in Freunds complete adjuvant. After absorption the specificity of the antisera was tested as described (9) using anti-C1 kindly supplied by Dr *R St oud* University of Alabama, Birmingham, USA.

Electroimmunoassay (10, 11) was used for the determination of C1 All analyses were performed with 0.075 M barbital buffer pH 8 6, containing 0 002 M EDTA. The concentration of antiserum in the gel was *0 25–1.5* per cent depending on the batch of antiserum used. Prior to electrophoresis serum or EDTA plasma was diluted 1/5 in electrophoresis buffer and 5 µl were applied without delay Electrophoresis was carried out at 3–4 V/cm for 18 hours The C1 values were given in percentage of the concentration in the reference standard pool which was said to be 100 per cent.

Results

C1 levels were determined in 100 normal sera. The mean value, the standard deviation, the median and the 95 per cent range are given in Table I

The logarithms of the concentrations were used for calculation of the 95 per cent range. The C1q and C1s values have been reported previously (13). The levels of C1q and C1s were compared with the C1r values obtained (Fig 1). The correlations between C1r and C1s (r = 0 768) and between C1r and C1q (r = 0 708) were comparable to the correlation found between C1q and C1s (r = 0 777) (13).

TABLE 1 *The Mean Standard Deviation Median and 95 Per Cent Range of C1r Levels in Normal Sera Determined by Electroimmunoassay Values Given in Percentage of a Normal Reference Pool*

Mean	S D	Median	95 % range
98	17	98	71–133

Double determinations of C1r in 10 sera were carried out repeatedly on the same and on different plates to assess the reproducibility of the method. The intraplate variation was 3.3 (SD) and the interplate variation 3 4 (SD)

The determinations of C1r in EDTA plasma gave inconsistent results. EDTA plasma often yielded higher C1r values than serum or gave rise to diffuse precipitates that precluded quantitative estimation. It should be pointed out that similar precipitation patterns would appear on analysis of serum, if samples were not applied promptly after dilution of serum in EDTA buffer.

Storage of serum for 5 days at room temperature did not influence the C1r values. On the other hand C1r values in EDTA plasma increased by 30-40 per cent already after 1 day of storage. Compared to serum EDTA plasma gave less distinct precipitates that became more diffuse on prolonged storage.

Repeated freezing and thawing did not appreciably affect C1r values in serum, but resulted in a moderate increase of the values in EDTA plasma.

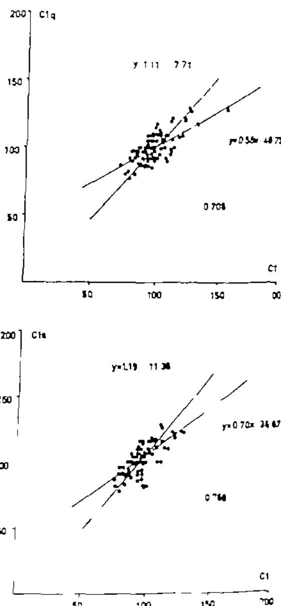


Fig 1 Levels of C1r compared with C1q (above) and C1s (below) in 100 normal sera. Numerical values used.

Discussion

The present study showed that C1r levels in human sera are readily determined with high reproducibility by electroimmunoassay. The C1r values did not change on storage of serum at room temperature which is of value for estimation of C1r levels in clinical work.

EDTA plasma stored at room temperature gave increasingly high and diffuse C1r precipitates. Apparently EDTA plasma should not be used for routine C1r determinations. The influence of storage on C1r in EDTA plasma might well be due to C1r activation in the presence of EDTA (14).

The correlation between the levels of C1q, C1r and C1s in normal sera most likely reflects the association of these proteins in the macromolecular C1 complex.

Selective C1r deficiency has been described in patients with SLE like syndromes or with glomerulonephritis (4 1?). An autosomal recessive mode of inheritance was suggested for the deficiency (3).

Moderately reduced C1r levels were found in SLE during the active phase (2). This is in accordance with our experience that patients with immune complex disease and reduced C1q and C1s levels also have low C1r values.

Elevated C1r and C1s and low C1q levels were reported in a few cases of SLE, rheumatoid arthritis and viral haemorrhagic fever (1). Selected sera from patients with chronic urticaria or hypogammaglobulinemia showing dissociation of C1

microcomponent levels were found to contain complexes between C1 and C1s (9)

The present investigation provides information for reliable quantitation of C1r by electroimmunoassay. Work is in progress to study the variation of C1r levels in disease in relation to other complement components.

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References. 1 Bracco M M E. de & Manni, J A Medicina (Bs Aires) 32 717 1972.—2. Bracco M. M. E. de Christian, C L. & Stroud R. M Clin. exp. Immunol. 16 453–459 1974 —3 Bracco M. M E. de Windhorst D Stroud, R. M & Moncada, B. Clin. exp. Immunol. 16 183–188, 1974 —4 Day N K., Geiger H Stroud R. M., Bracco M M E. de Windhorst D., Moncada, B. & Good R A J clin. Invest. 51 1102 1108, 1972.—5 Gaarot, P O.. Scand. J clin. Lab. Invest. 29 Suppl. 124 83–88, 1972.—6. Laurell A B., Lindegren J., Malmros I & Mårtensson, H.: Scand. J clin. Lab. Invest. 24 221–225 1969 — 7 Laurell, A -B., Sjöholm A & Johnson U.: Clin. exp. Immunol. 7 423–430 1970.—8. Laurell A -B. Mårtensson, U & Sjöholm, A G Clin. Immunol. Immunopathol. 5 308–313 1976.—9 Laurell, A. B Mårtensson U & Sjöholm A G.. Acta path microbiol. scand. Sect. C, 84 in press, 1976.—10. Laurell, C -B.. Analyt. Biochem. 15 45–52, 1966.— 11 Laurell, C.-B.. Scand. J clin. Lab. Invest. 29 Suppl. 124 21–37 1972.—12. Pickering, R. J., Naff G. B., Stroud R. M., Good, R A & Gewurz, H J J exp. Med. 141 803–815 1970 —13 Sjöholm, A G Scand. J Immunol. 4 25–30 1975 — 14 Zaccardi R. J & Cooper N R.. J Immunol. 116 496–503 1976.

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PRECIPITINS IN LIBERIAN SERA REACTING WITH GOAT AND SHEEP SERA

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Willcox, M Precipitins in Liberian sera reacting with goat and sheep sera. Acta path. microbiol. scand Sect C, 84 427–428, 1976.

Precipitins in nine sera from normal Liberian adults were shown to react with an α-globulin in goat and sheep sera. No cross-reaction was demonstrated with bovine sera Hence these precipitins are different to others previously described. At present their significance, if any is unknown They are important as a possible cause of unexplained reactions in serological tests using sheep or goat serum as a source of antigen or antibody

Key words Liberian sera precipitins.

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In a recent study of anomalous reactions when using a commercial kit for screening for hepatitis-B surface antigen (HB_sAg) a number of Liberian sera were found to contain precipitins reacting with goat and sheep sera (10) Full investigation of these precipitins was not possible because of the small volumes of sera available. However preliminary studies were made and are described here.

Material and Methods

The nine sera described were found positive among a total of 214 specimens from Liberian

blood donors sent to this laboratory for HB_sAg screening

Sera were tested for HB_sAg by counter-electrophoresis (CEP) (3) and a commercial haemagglutination test (Hepanosticon, Organon Teknika) and for rheumatoid factor by both a latex slide test (RA Test, Hyland) and a Waaler Rose method (5)

Standard techniques were used for double radial immunodiffusion (9) and immunoelectrophoresis (4) Immunoglobulin levels were estimated by single radial immunodiffusion (8) CEP with varying inter well distances was used to react the Liberian sera with goat serum.

Results

All nine Liberian sera were negative for HB_sAg and none contained rheumatoid factor

In immunodiffusion tests the nine sera produced precipitin bands when reacted with both goat and sheep serum. These bands gave reactions of identity with each other but not with those produced against goat and sheep serum by a rabbit anti sheep-γ-globulin antiserum. Reactions of partial identity were shown, however when precipitin bands produced by the nine Liberian sera were compared with those given by a rabbit anti-goat globulin serum. None of the nine sera produced precipitin bands when reacted against cow horse guinea pig rabbit or human serum. Seven sera were tested by immunoelectrophoresis. The sera were, after electrophoresis, reacted with horse anti human serum, rabbit anti human IgG IgM and IgA monovalent antisera and whole goat or sheep serum. In each case the fraction of Liberian serum reacting with goat and sheep sera had the same mobility as IgM Goat or sheep serum was after electrophoresis reacted with rabbit anti-goat/sheep-serum antiserum or Liberian serum. In this way the fraction of goat or sheep serum reacting with the Liberian sera was shown to be an α-globulin.

No reaction was obtained by CEP using a 7 mm inter well distance with the seven Liberian sera in the anodal wells and goat serum in the cathodal. However by increasing the inter well gap to 20 mm, in each case a precipitin band appeared close to the anodal well.

By the Mancini technique using rabbit antiserum in the gels, immunoglobulin levels of the seven sera tested were IgG 15–50 g/l (mean 23 6 ± 12.3) IgM 0 96–4.2 g/l (mean 1.9 ± 0 99) and IgA, 0 8–2.5 g/l (mean 1 4 ± 0.65)

Discussion

The precipitins in the nine Liberian sera appear to be identical They demonstrated a similar electrophoretic mobility to IgM. However on the data available it is not possible to exclude the presence of IgG precipitins Full analysis must await fresh samples. Nevertheless, these precipitins are clearly different to the anti-ruminant antibodies described earlier (1 6 7) The latter reacted with IgG or IgM in the ruminant serum whereas the present precipitins are active against an α-globulin. The early antibodies also cross-reacted with bovine serum. They were in addition often associated with an IgA deficiency whereas the IgA levels of the sera described here were within the range of values found for normal Liberians in a previous study (2)

The precipitins appear to be not uncommon in Liberian sera but were not detected in over 500 Swedish sera tested in this laboratory Their cause is unknown as yet. One possibility is that they may in some way be related to diet. Another is that they appear as a non-specific responce to parasitic or other infection. Their present importance is as a cause of anomalous reactions when using goat or sheep serum reagents for precipitin and other serological tests on Liberian and similar populations. An exception to this may be CEP with normal well distances because of the rapid anodal mobility of the reacting fraction of the goat or sheep serum.

References 1 *Alter H J Polesky H F & Holland P V* J Immunol. *108* 358–369 1972.—2 *Cappuccinelli P Frentzel-Beyme R R., Martinetto P & Sena L.* J Trop. Med. & Hyg. *75* 180–182 1972.—3 *Coombridge B S & Shaw C* Lancet *ii* 1184–1185 1971 —4 *Grabar P & Williams C A Jr* Biochim. Biophys. Acta *10* 193–194 1953 —5 *Houba, V & Allison A C.* Lancet *i* 848–852 1966 —6. *Huntley C C., Robbins J B Lyerly A D & Buckley R H.* N Engl Journ. Med. *284* 7–10 1971 —7 *Lehkola, J & Vyas G N* J Lab. Clin. Med. *77* 629–638 1971 —8. *Mancini, G., Carbonara, A. D & Hermans J F* Int. J Immunochem. *2* 235–254 1965 —9 *Ouchterlony Ö* Progress Allergy *5* 1–77 1958 —10 *Willcox M C* Acta path. microbiol scand Sect. B, *84* 115–116 1976.

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ANTIGENIC PROPERTIES OF A DNA PREPARATION FROM CALF THYMUS USED FOR THE DEMONSTRATION OF ANTI DNA

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Jonsson, J & Norberg, R. Antigenic properties of a DNA-preparation from calf thymus used for the demonstration of anti-DNA. Acta path. microbiol. scand Sect. C, 84 429–441 1976.

It was attempted to evaluate passive haemagglutination of antigen coated, tanned erythrocytes as test by which to demonstrate anti-DNA in systemic lupus erythematosus. The antigen was prepared using minimum of procedures in order to produce a native preparation. The resulting material had most of the criteria applying to nativ DNA, but the protein content was about 9 per cent. It contained thymocyte specific component, but no demonstrable trace of bovine species antigen. The reactions between the antigen and an anti-DNA serum from a patient with suspected SLE were inhibited by DNA and DNA-histone, but not ppreciably by ENA, RNA or deoxyribonucleosides. Passiv haemagglutination reactions against the antigen were positively correlated to a homogeneous immunofluorescence nuclear pattern and negatively correlated to speckled pattern. Passive haemagglutination titres against ENA and DNA antigen were not correlated. Seventy-three per cent of randomly selected sera gav either purely DNase sensitive reactions (19 per cent) or reactions of combined sensitivity to DNase and other enzymes. Twenty-eight out of 53 sera reacting in the pass haemagglutination test reacted also in the immunofluorescence test against *Chrithidia luciliae* kinetoplasts. The latter reactions were DNase sensitive. It pplies to both tests that DNase sensitive, but RNase resistant, reactions were well correlated, irrespectiv of their sensitivity to trypsin hile DNase resistant or DNase and RNase sensitiv reactions were not correlated. The passiv haemagglutination test using nativ but relatively crude DNA-preparation coated on tanned sheep erythrocytes supplemented by specificity tests with DNase and RNase treated antigen gives about the same information as the indirect immunofluorescence test against *Chrithidia luciliae* kinetoplasts. Furthermore, the results show that patients sera reacting with homogeneous nuclear pattern in the indirect immunofluorescence test may contain not only anti-DNA and anti-nucleohistone antibodies, but also antibodies to a number of non-histone chromatin associated proteins some of which contain RNA.

Key words DNA-preparation antigenic properties calf thymus anti-DNA.

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Sera from patients with connective tissue disease react with two main groups of nuclear antigens i.e. those giving a speckled and those giving a homogeneous nuclear pattern in the indirect immunofluorescence test (IFL). The

former are apparently constituents of the nucleoplasm and are extractable by physiological buffers. The latter are not extractable and more or less firmly associated with the chromatin.

Several of the antigens are of diagnostic importance. For example antibodies to a soluble ribonucleoprotein (sRNP 15 19) in the extractable fraction is considered to be connected with mixed connective tissue disease (MTCD) or systemic sclerosis (SS) Antibodies to another antigen in the same fraction, the Sm antigen (15 25) is according to present knowledge only found in systemic lupus erythemathosus (SLE) The most important non-extractable antigen is the native double helix of desoxyribonucleic acid (DNA)

Antibodies to this latter antigen seem to be specific of SLE. Their presence is prognostically unfavourable and their titre follows the disease activity The demonstration of DNA-antibodies is thus of considerable clinical importance. The passive haemagglutination test using DNA coated tanned sheep erythrocytes has been used for this purpose in our laboratory for a considerable period, although the difficulties in obtaining active, native and pure DNA preparations (see Discussion) have been recognized. In determinations of DNA-antibodies, the use of the haemoflagellate *Chrithidia luciliae* (1) proved to be an easy means by which the tanned cell test could be evaluated as a diagnostic procedure. A comparison of the two methods will be presented in this report

The DNA of the antigen preparation used in the present study was apparently of a highly native nature, but it was admixed with a number of other antigens, mainly proteins. These substances are of methodological interest since they might give rise to diagnostic errors. Reactions against these substances also signify the existence of antibodies to chromatine-associated antigens other than histone. The results of the enzyme studies described in this report attest to multiplicity of chromatine-associated antigens some of which may be important, theoretically as well as from the point of view of diagnostic specificity

MATERIALS AND METHODS

Antigens

Tissue Sections

Cryostat sections from thyrotoxic human thyroid glands were used unfixed fixed with acetone or with equal parts of methanol and acetone for 5 minutes. The thickness of the sections was 8 μ.

Nuclear Fractions

Domestic preparations. Extractible nuclear antigen (ENA) was prepared as described by *Sharp et al.* (19 20) The undissolved material left after this procedure was further washed and dissolved in 1 M NaCl This solution was cleared by centrifugation and the nucleoprotein was precipitated by making the solution 0 15 M. The DNA was then prepared by redissolving the precipitate in 1 M NaCl injecting the solution into ethanol and dissolving in 0 15 M NaCl as described by *Signer & Schwander* (17 21)

The coated cells were used without further treatment and after treatment with DNase, RNase and trypsin as described below

Commercial preparations. The following purified nucleic acid preparations were purchased from Sigma chemical Co St Louis MO U.S.A

	Sigma Cat.	N r
DNA type I from calf thymus	D	1501
RNA type VI from Tourla yeast	R	6625
S-RNA type IV from calf liver	R	7250
2-deoxy guanosine 5 monophosphoric-acid	D	9500
2-deoxy cytidine 5 monophosphoric-acid	D	7750
2-deoxy adenine 5 monophosphoric acid	D	6?50

Calf thymus DNA was also purchased from Worthington Co USA Cat code DNA 2105

The preparations were used as antigens in the passive haemagglutination mixed haemadsorption and haemagglutination inhibition tests.

Chrithidia luciliae

Smears from suspensions of cultured protozoa fixed with ethanol were kindly supplied by drs *Feltkamp & 4 den* Amsterdam (1 2, 22)

Antisera

Patients Sera

Patients sera sent in for the diagnosis of antinuclear antibodies and showing a homogeneous nuclear immunofluorescence pattern in the dil tion 1/100 or more ere further analysed by passive haemagglutination tests against ENA and DNA. If positive, their reactivity against enzyme treated ENA and DNA coated erythrocytes was examined.

On the basis of these examinations, the sera was classified into 6 different types the characteristics of which appear from the tables.

The following sera were prepared by repeated intramuscular injections. The cellular antigens were given without adjuvant. The soluble antigens were given in Freunds complete adjuvant.

Specificity	Animal immunized	Antibody titre
Anti-bovine thymocyte	rabbit	1/2560
Anti-guinea-pig thymocyte	rabbit	1/2560
Anti-HeLa cell	rabbit	1/1600
Anti-bovine whole serum	rabbit	1/40960
Anti-bovine γ-globulin	rabbit	1/2560
Anti-bovine albumin	rabbit	1/20480
Anti-human-lymphocyte	horse	1/2000

Enzymes

Deoxyribonuclease DNase I was purchased from Sigma Chemical Co., St Louis, USA. (Stock No. DN-CL, Lot 33C 0223) The enzyme is supposed to act preferentially on nati e d-DNA (7).

Ribonuclease RNase IA was also purchased from Sigma Chemical Co (Product No. R4875 Lot 72C-1440).

Trypsin A 1 per cent solution of Trypsin 1 300 (National Biochemical Corp Clev 1 Ohio, USA) was used.

METHODS

Passive Haemagglutination T sts

The passive haemagglutination test (5) was used to demonstrate antibodies to DNA and extractable nuclear antigen (ENA). Sheep erythrocytes ere tanned and coated as described by *Sharp et al.* (19 20). Sera were titrated on plastic trays according to the micro titre system (18, 19 24).

Haemagglutination I hibition T sts (8)

These tests were performed by making 0.25 ml twofold serial dilutions of antigen in serological tubes, 4 agglutinating units of anti-DNA serum being added to the diluent. The tubes were left at room temperature for 2 hours before 0.05 ml of 2 per cent suspension of DNA-coated erythrocytes was added, as described above.

Immunofluorescence Tests

The indirect immunofluorescence test was performed according to the standard procedure used as diagnostic routine in this laboratory (4 26). The fluorescein isothiocyanate antihuman globulin conjugate used had an antibody content of 2.6 mg/ml and an F/P quotient of 4.8 × 10⁻ (4). The preparations were read by transmitted light in a Zeiss immunofluorescence microscope with an HBO 200 L 2 mercury lamp a dry darkfield condenser exciter filter BG 3 and occular filter 44.

Enzyme T atments (19 20)

T eatment with DNase or RNase Sheep erythrocytes tanned and coated as described were sedimented by centrifugation and resuspended to a 20 per cent suspension in phosphate buffered saline (PBS) containing Ca^{++} (0.9 mM/1) and Mg^{++} (0.4 mM/1). To this suspension was added an equal part of enzyme solution (see below). The mixture was incubated t +37 C for 60 minutes and then centrifuged and washed and resuspended as a 2 per cent suspension in PBS without Ca^{++} and Mg^{++}. It was kept at this stage for 15 minutes t room temperature. The medium was then changed to barbital buffer with 1 per cent of normal rabbit serum (NRS) before use in the titrations.

Slides with smears of *Crithidia luciliae* fixed for 10 minutes with methanol were immersed for 30 minutes in PBS pH 7 4 containing Ca^{++} (0.9 mM/1) and Mg^{++} (0 4 mM/1) and 800 Kunitz units/ml of DNase or RNase, respecti ely. They were then rinsed rapidly three times with PBS w thout Ca^{++} and Mg^{++} in the course of 5 min, dried and used in the immunofluorescence test. The enzyme solutions were prepared by dissol ing the commercial powders in PBS pH 7 4 with Ca^{++} and Mg^{++}. The solutions used were adjusted to contain 400 and 800 Kunitz units of DNAse and RNAse per ml, respectively.

T eatment with trypsin. Erythrocytes tanned and coated with DNA or ENA as described were sedimented by centrifugation and resuspended to a 10 per cent suspension in PBS containing Ca^{++} and Mg^{++} and 0 25 per cent trypsin (SBL). This mixture was left at 37 C for 60 minutes. The erythrocytes were then handled as those treated with DNase and RNase.

Slides with smears of *Crithidia luciliae* were treated as described for DNase or RNase but 0 25 per cent solution of trypsin was used instead of the former tw enzymes.

Chemical Methods

Deoxypentose and purine bound deoxypentose were estimated by the Dische diphenylamine method as modified by *Davidson et al.* (6, 7)

Pentose and purine bound pentose were deter

mined by the orcinol method as described by *Schneider* (16)

Phosphorous was estimated according to the method described by *Allen* (3)

Protein was estimated by the methods described by *Lowry et al.* (14) and *Kjeldahl* (13)

Direct ultraviolet spectrophotometry was used as a preliminary test for nucleic acids.

RESULTS

A The DNA-antigen

1 Physical properties The material resulting from the procedure described was precipitated by ethanol as 3–10 cm long fibres easily wound up on a glass rod and leaving a clear matrix. The fibres were easily and completely dissolved in PBS pH 7 4 producing a very viscous clear uncoloured solution. The material was stored as lyophilized fibres. After 6–12 months in this state at +4° C no change in immunological or physical properties was noted when it was redissolved

2 Stoikiometric analysis The contents of desoxyribose, ribose phosphorous, total nitrogen and total protein of the DNA antigen were determined and compared with the composition of a standard DNA preparation and with the calculated composition of DNA (Table 1)

The domestic preparation was found to contain about 9 per cent protein and had a low atomary nitrogen/phosphorous quotient. Its ribose contents were lower than those of the standard preparation. Incidentally the latter was not either completely free of protein.

The ultraviolet absorbance curve of the domestic preparation had a maximum at the expected wave length and its shape indicated a considerable degree of purity (Fig 1)

3 Immunochemical analysis A number of sera with known specificity were tested against the antigen (Table 2) Among the sera from patients with connective tissue diseases none of those giving an entirely speckled nuclear immunofluorescence pattern has so far been found to react with the DNA antigen. Sera reacting with that antigen gave a homogeneous nuclear immunofluorescence pattern. Sera giving a speckled nuclear pattern and a reaction with the DNA-antigen have so far always had a homogeneous component of the nuclear staining Many sera reacting in high titre with ENA did not react with the DNA antigen. This was found to apply to sera presenting all of the established ENA reactivity patterns (10 11)

Anti-bovine thymocyte and anti-guinea pig thymocyte sera reacted weakly with the DNA antigen Anti human lymphocyte, anti-HeLa cell, anti bovine γ-globulin and anti-bovine albumin sera did not react (Table 2) Nevertheless, a reaction was obtained by anti bovine whole serum.

Haemagglutination inhibition tests were

TABLE 1 *Composition of DNA Antigen*

Substance		Total protein* w/w %	Total nitrogen§ w/w %	Total phosphorus† w/w %	Atomary N/P quotient	Ribose‡ w/w %	Deoxyribose w/w %
DNA	calculated	0	15 24	9 22	3 65	0	39.33
	found, experimental	8 62	8 97	9 00	2 20	1 00	11.20
	found, standard	1 30	10 60	5 90	4 11	2.00	17.60

* Method of *Lowry*
§ Method of *Kjeldahl*
† Method of *Allen*
‡ Orcinol method
Diphenylamine method

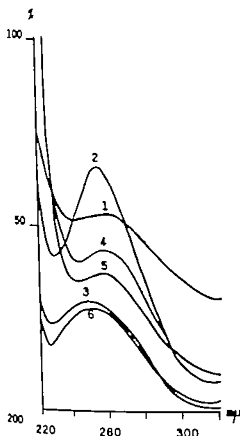


Fig. 1 Ultraviolet absorbance curves of experimental DNA and ENA preparations compared with the curves of standard DNA and RNA.
1 Experimental DNA-protein 1 mg/ml.
2 Experimental DNA 0 1 mg/ml
3 Standard DNA (Sigma) 0 02 mg/ml
4 Experimental ENA I 1 mg/ml
5 Experimental ENA II 1 mg/ml
6 Standard RNA (Sigma) 0.01 mg/ml

performed in the DNA-anti-DNA system using a collagenous serum where agglutination with the DNA-antigen was inactivated by DNAse. Inhibition was produced by very low concentrations of domestic DNA, by low concentrations of DNA-protein and by moderately high concentrations of ENA. RNA caused no demonstrable inhibition. 2 desoxyguanylphosphate was slightly inhibitory while 2-desoxycytidylphosphate and 2 desoxyadenylphosphate were not (Table 3)

TABLE 2. *Reactions Produced by Various Antisera against Tanned Cells Coated with Desoxyribonucleic Acid (DNA)*

Specificity of antiserum	Antigen Desoxyribonucleic acid DNA
Anti-ENA	<10
Anti-DNA	640
Anti-bovine thymocyte	40
Anti-guinea pig thymocyte	20
Anti-human lymphocyte	<10
Anti-HeLa cell	<10
Anti-bovine whole serum	320
Anti-bovine γ-globulin	<100
Anti-bovine albumin	<10

B. Types of Reactivity Distinguished by the Passive Haemagglutination Test

Six different types of reactivity of sera could be distinguished by passive haemagglutination against DNA antigen on tanned sheep erythrocytes by testing against non-enzyme treated cells and cells treated with DNase, RNase and trypsin respectively

Type 1 sera gave a reaction that would be inhibited by DNase but remained unaffected or was enhanced (Table 4) by RNase or trypsin. About 19 per cent of the sera reacted in this way (Table 5)

Type 2 sera gave a reaction that was inhibited by DNase or trypsin but not by RNase (Table 4) About 21 per cent of the sera belonged to this group (Table 5)

Type 3 sera gave a reaction that was inactivated by DNase or RNase but not by trypsin. This type comprised about 9 per cent of the sera (Table 5)

Type 4 sera gave reactions that were inactivated by DNase, RNase or trypsin (Table 4) About 24 per cent of the sera gave this type of reaction (Table 5)

Type 5 sera gave reactions that were inactivated by trypsin but not by DNase or RNase (Table 4) This type of reaction was given by about 5 per cent of the sera (Table 5) DNase and RNase often enhanced the reactions (Table 6)

Type 6 sera gave reactions that remained

TABLE 3 *Inhibiting Effects of Various Nuclear Fractions on the Passive Haemagglutination of DNA antigen Coated Tanned Sheep Erythrocytes by an Anti-DNA Serum*

Specificity of reacting serum	Aggl units	Antigen used for haemagglutination inhibition	Least inhibiting conc. (μg/ml) in haemagglutination against DNA-antigen
Anti DNA	4	ENA*	130
		DNA*	0 2
		RNA	>1000
		DNA prot*	20
		2 dx GP	250
		2 dx CP	>500
		2 dx AP	>500

* Domestic preparations.

unaffected or often were enhanced (Table 4) by all three enzymes. The reaction was noted to occur in about 22 per cent of the sera (Table 5)

C Correlations of Haemagglutination Test with Other Tests

1 Immunofluorescence test against mammalian cell nuclei A reaction with the DNA antigen was positively correlated to the homogeneous nuclear immunofluorescence pattern and negatively correlated to the speckled pattern produced by the indirect immunofluorescence test against cell nuclei in human thyroid tissue sections (Table 7) Conversely a reaction with the ENA was positively correlated to the speckled pattern and negatively correlated to the homogeneous pattern (Table 8) In agreement with this, a correlation diagram where anti DNA is plotted versus anti ENA (Fig 2) shows two clearly unrelated titre groups.

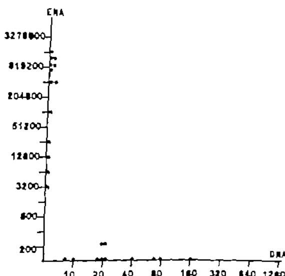


Fig 2 Correlation diagram of haemagglutination titres obtained by ENA and DNA antigen, respectively

Immunofluorescence test against Chrithidia luciliae. Fifty three patients sera reacting in the passive haemagglutination test against DNA antigen were also examined by way of the indirect immunofluorescence test against *Chrithidia luciliae* kinetoplasts. The Chrithidia smears were examined untreated and pretreated by the same enzymes as in the passive haemagglutination tests i.e. DNase, RNase and trypsin.

Twenty-eight of the patients sera were found to react with Chrithidia kinetoplasts. All these reactions were inactivated by DNase while in the passive haemagglutination test about 30 per cent of sera were DNase resistant (Table 9) Sensitivity to DNase as well as to trypsin was found to range at about the same low frequency (10 per cent) in both tests Sensitivity to DNase and RNase was also found by both tests (Table 9) Sensitivity to DNase only was the usual finding to be obtained by the Chrithidia test (75 per cent) while this type of reactivity was more rare in the passive haemagglutination

TABLE 4 *Different Reactivity Patterns Encountered among Sera Reacting with an Experimental DNA preparation in the Passive Haemagglutination Test*

	ANF	Reaction against DNA: Untreated	DNase treated	RNase treated	Trypsin treated	Reaction against RNA: Untreated	DNase treated	RNase treated	Trypsin treated
DNase sensitive only	400 H	640	<10	160	640	<200	<200	<200	<200
	800 HKN	640	<10	80	1280	5200	102400	102400	51200
DNase and trypsin sensitive	800 H	160	20	320	20	<200	<200	<200	<200
	800 H	640	<10	640	<10	25600	6400	200	<200
DNase and RNase sensitive	100 H	160	<10	<10	2560	<200	<200	<200	<200
DNase RNase and trypsin sensitive	400 H	160	<10	20	<10	200	<200	<200	200
	400 H	80	<10	10	<10	12800	25600	102400	12800
Trypsin sensitive only	400 H	80	320	160	<10	400	400	200	<200
		160	320	640	<10	1600	1600	1600	400
Resistant to tested enzymes	400 H	20	160	160	40	<200	<200	<200	<200
	100 H	20	80	80	320	200	200	400	<200
	100 HK	160	640	320	1280	102400	102400	1600	1600

TABLE 5 *Distribution of Types of Reactivity Types over Sera Reacting with a Domestic DNA Preparation*

	%
DNase sensitive only	19
DNase and trypsin sensitive	21
DNase and RNase sensitive	9
DNase RNase and trypsin sensitive	24
Total DNase sensitive	73
Trypsin sensitive only	5
Resistant to tested enzymes	22
Total DNase resistant	27
Total	100

Total number examined 63

test. Sensitivity to all of the three enzymes respectively did not occur if the Chrithidia test was used It applies to both tests that DNase sensitive but RNase resistant reactions were well correlated irrespective of sensitivity to trypsin (Fig 3 a) On the other hand, DNase resistant or DNase – RNase sensitive reactions were poorly correlated to the reactions of the same sera obtained by the Chrithidia test (Fig 3 b)

Among the 25 sera which reacted in the passive haemagglutination test but not in the Chrithidia test, 10 gave DNase sensitive reactions. Only one of these was exclusively DNase sensitive (group 1 Table 9) Another was DNase and RNase sensitive (group III, Table 9) The remaining 8 were sensitive

TABLE 6 *Number of DNA Haemagglutination Reactions Enhanced Two or More Titre Steps by Enzyme Treatment*

	Passive haemagglutination anti DNA titre enhanced by			
	DNase	RNase	Trypsin	Total examined
DNase sensitive only	0	3	2	12
DNase and trypsin sensitive	0	1	0	13
DNase and RNase sensitive	0	0	4	6
DNase RNase and trypsin sensitive	0	0	0	15
Trypsin sensitive only	2	1	0	3
Resistant to all tested enzymes	3	5	5	14
Total	5	10	11	63

TABLE 7 *Relationship between DNA Haemagglutination Test and Nuclear Immunofluorescence Patterns*

		IFL		
		Homogeneous ≥1/10	Speckled ≥1/10	Total
DNA test	<1/10	6	15	21
	≥1/10	13	4	17
Total		19	19	38

P denoting correlation between a homogeneous nuclear pattern and a positive anti DNA test <0 05 (χ^2 test)
P denoting correlation between a speckled nuclear pattern and a negative anti DNA test <0 05 (χ^2 test)

TABLE 8 *Relationship between ENA Haemagglutination Test and Nuclear Immunofluorescence Pattern*

	IFL		
	Homogeneous ≥1/10	Speckled ≥1/10	Total
ENA <1/200	10	2	12
test ≥1/200	9	15	24
Total	19	17	36

P denoting correlation between a speckled pattern and positive anti-ENA test $0.01<P<0.02$ (X^2-test)
P denoting correlation between a homogeneous pattern and a negative anti-ENA test <0.05 (X^2-test.

to all three enzymes (group IV Table 9)

Each of the three enzymes tested could either reduce or enhance the reaction in either of the two tests. A reduction or a complete disappearance of the reaction was the usual effect of DNase treatment. Treatment with the other two enzymes, especially trypsin, had often an enhancing effect in both tests. Quite often however the reducing or enhancing effects in the two tests might not be associated (Table 10)

DISCUSSION

The demonstration of antibodies against DNA is recognized as an important diagno-

TABLE 9 *Distribution of Reactions over the Six Groups Presenting Different Enzyme Sensitivity Patterns in the Passive Haemagglutination Test*

Group	Untreated	Reactivity after enzyme treatment			Supposed antigen	Passive haemagglutination against DNA antigen %	Immunofluorescence test against *Chrithidia luciliae* %
		DNase	RNase	Trypsin			
I	+	—	+	+	DNA	18.9	75.0
II	+	—	+	—	DNA-protein	11.3	7 1
III	+	—	—	+	RNA?	22.6	17.9
IV	+	—	—	—	RNA-protein	15.1	0
V	+	+	+	—	Protein	3.8	0
VI	+	+	+	+	Non-trypsin sensitive protein	28 3	0
Total number examined						53	28

— denotes reaction eliminated by enzyme treatment of antigen.
+ denotes reaction in the presence of untreated antigen or persisting after enzyme treatment of antigen

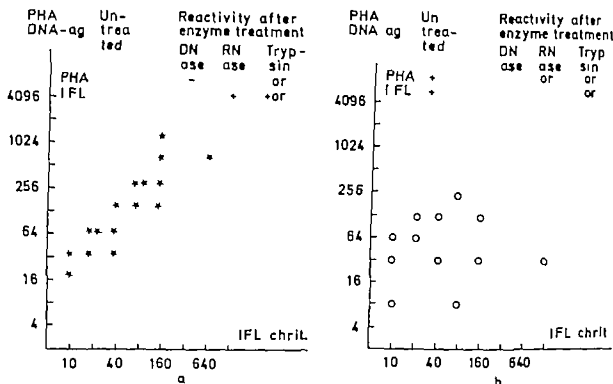


Fig 3 Different degrees of correlation between titres obtained by different types of sera against DNA in the passive haemagglutination (PHA) and Chrithidia (IFL chrit) tests. Reactions against DNase sensitive RNase resistent antigen are shown to the left (Fig 3 a) Reactions against DNase resistent and/or RNase sensitive antigen are shown to the right

stic procedure in the management of connective tissue disease Several laboratory methods have been developed for this purpose the most important being the Farr test, (2) the Chrithidia test (1) and the passive haemagglutination test (19) Using the Chrithidia test native DNA is demonstrated *in situ* in the kinetoplast of the Chrithidia organisms. As regards the use of the latter test, the experience gained is only limited Still, its serological specificity is apparently high, but it may be rather insensitive and it is a disadvantage that it has to be read microscopically The other two tests utilize well-known, highly reliable serological techniques, but they depend on isolated DNA preparations, the

TABLE 10 *Titre Changes against DNA Induced by Enzymes in the Passive Haemagglutination and Immunofluorescence Tests*

Enzyme	Passive haemagglutination test against DNA antigen			Indirect immunofluorescence test against *Chrithidia luciliae* kinetoplasts		
	Number examined	% of reactions Reduced	% of reactions Enhanced	Number examined	% of reactions Reduced*	% of reactions Enhanced
DNase	56	66 1	5 4	26	100 0	0 0
RNase	54	37 0	20 4	31	16 1	3.2
Trypsin	54	18 5	18.5	27	11 0	18.5

* To be recorded as reduced or enhanced a reaction had to show a titre change of at least two twofold dilution steps.

qualities of which usually are unsatisfactory

The properties of a DNA-preparation may be judged from its purity and degree of denaturation. These two criteria are to some extent antagonistic since purification from RNA and protein usually has to be effected at the cost of the nativity of the preparation. No doubt, the latter factor is the more important since reactions with contaminants can, for the most part, be controlled by supplementary procedures, including enzyme treatment of the antigen.

The experimental DNA preparation used in the present study consisted predominantly of DNA as judged from the stoikiometric analysis and it contained only insignificant quantities of RNA. However its high relative protein contents suggest a considerable admixture of protein. The high content of phosphorous, near that to be expected in the case of pure DNA in combination with a depressed nitrogen/phosphorous ratio may indicate that a considerable proportion of the protein was phosphorylated non-histone chromatine protein. It is therefore possible that part of the trypsin sensitive antigens reacting in the passive haemagglutination test may belong to this class of proteins. Another part may be histone residues left on the DNA molecules. Indeed, short peptide residues left on the DNA-molecules would appear to be a prerequisite for the attachment of the antigen to the tanned sheep erythrocytes.

No extranuclear antigenic components were found in the DNA preparation and any species specific components could not be reliably demonstrated. The weak reaction with anti-bovine thymocyte serum was probably organ specific since a reaction with anti-guinea pig thymocytes serum was also noted. The reaction obtained by anti-bovine whole serum may also be due to a thymogenic component present in the serum and injected into the rabbits during immunization.

The complexity of the domestic DNA antigen and the multitude of antibody-specificities to DNA-associated antigens was clearly demonstrated by the enzyme studies. At least five specificities in the preparations were distinguished by this procedure. Four reactivity types were inactivated by DNase I from calf pancreas, an endonuclease preferentially reacting with native DNA. It is therefore probable that these types of reactivity depended at least in part, on the native double helix configuration. This was also supported by the fact that DNase sensitive haemagglutination reactions were closely correlated with reactions against *Chrithidia luciliae* in the indirect immunofluorescence test. One of the four types of haemagglutination was inactivated by DNase alone. This type, although least common, may be the only single anti-DNA reaction. Still, also the types in which the DNase sensitivity was combined with sensitivity to other enzymes correlated well to the Chrithidia test. In some cases, the reactivity was enhanced by RNase or trypsin treatment as if the latter caused a better exposure of some determinants. It should be noted that corresponding phenomena occasionally occurred in the Chrithidia test (12)

The most common type of passive haemagglutination reaction was the one that was inactivated both by DNase and trypsin and therefore was suspected to be due to a soluble desoxyribonucleo-protein (sDNP 24) Haemagglutinations to be inactivated by DNase and RNase may be explained if it is assumed that RNA – DNA complexes sometimes form a new common determinant. This may also occur in the Chrithidia test (12)

The degree of denaturation is the major factor to decide the choice of type and purity of a DNA preparation. The antigenicity of the DNA molecules seems to depend entirely on the steric configuration of the double helix, especially on the configuration of the sugar phosphate backbone of the adenine-thymidine nucleotide sequences (23) This antigenically specific configuration is lost during the steric change induced by denaturation.

In the native stage, the sugar pyrimidine and -purine bases of the double helix are not exposed as they are on single stranded molecules. Single stranded parts along the double helix may be due to *in vivo* transla-

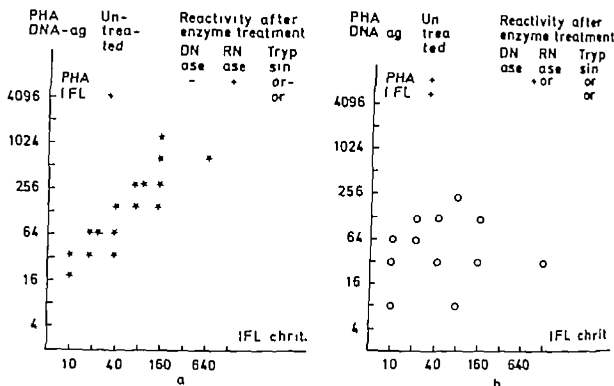


Fig 3 Different degrees of correlation between titres obtained by different types of sera against DNA in the passive haemagglutination (PHA) and Chrithidia (IFL chrit) tests. Reactions against DNase sensitive RNase resistent antigen are shown to the left (Fig 3 a). Reactions against DNase resistent and/or RNase sensitive antigen are shown to the right.

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TABLE 10 *Titre Changes against DNA Induced by Enzymes in the Passive Haemagglutination and Immunofluorescence Tests*

Enzyme	Passive haemagglutination test against DNA-antigen			Indirect immunofluorescence test against *Chrithidia luciliae* kinetoplasts		
	Number examined	% of reactions Reduced*	Enhanced	Number examined	% of reactions Reduced*	Enhanced
DNase	56	66 1	5 4	26	100 0	0.0
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acting with cell nuclei in the immunofluorescence test. Scand. J. Rheum. 5 177–183 1976.

11 *Jensen J Steiner M & Klein, E.*, An extractable nuclear antigen not attaching to tannic acid treated erythrocytes. Clin. exp. Immunol. 25 144–151 1976.

12 *Jensen, J & Norberg R.* Effect of enzyme treatment of *Crithidia luciliae* on the reactivity of its kinetoplast with anti-DNA sera. In press. Scand. J Rheum. 1976.

13. *Kjeldahl, J* Neue Methode zur Bestimmung des Stickstoffs in organischen Körpern. Zeitschrift f. Anal. Chemie 22 366–382, 1883

14. *Lowry O H Rosebrough N J., Farr A. L. & Randall, R. J.*: Protein measurement with the Folin phenol reagent. J Biol. Chem. 193 265–275 1951

15. *Northway J D & Tan E. M.*, Differentiation of antinuclear antibodies giving speckled staining pattern in immunofluorescence. Clin. Immunol. & Immunopath. 1 140–154 1972.

16. *Schneider W C* Determination of nucleic acids in tissues by pentose analysis In *Colowick, S P & Kaplan, N O* (Eds.) Methods in enzymology Vol. 3 N Y Acad. Press 1957 pp. 680–684

17 *Schwander H & Signer R* Darstellung von hochmolekularem Natrium thymonucleat aus Kalbthymus. Helv Chim. Acta 33 1521–1526, 1950.

18. *Sever J L* Application of microtechnique to viral and serological investigations. J Immunol 88 320–329 1962

19. *Sharp G Irvin W., Tan E., Gould G & Holman, H* Mixed connective tissue disease An apparently distinct rheumatic disease syndrome associated with a specific antibody to an extractable nuclear antigen (ENA) Am. J Med. 52 148–159 1972.

20 *Sharp G C., Irvin W S., La Roque Robert L., Velez Carmen Daly Virginia, Kaiser A. D & Holman H.*, Association of autoantibodies to different nuclear antigens with clinical patterns of rheumatic disease and responsiveness to therapy J Clin. Invest. 50 350–359 1971

21 *Signer R. & Schwander H.*, Isolierung hochmolekularer Nucleinsäure aus Kalbthymus. Helv Chim. Acta 32 853–859 1949

22. *Steinert M.*, L'absence d'histone dans le kinétonucléus des trypanosomes. Étude cytochimique Experimental cell research. 39 69–73 1965.

23 *Stollar B. D* The detection of anti-DNA and DNA/anti-DNA complexes. In *Feltkamp T E. W* (Ed.) The significance of the determination of anti-DNA and DNA/anti-DNA complexes. Proc. 1st Bertine Coperberg. Conf. Scand. J Rheum. Suppl. 11 7–11 1975.

24 *Takatsy G.*, The use of spiral loops in serological and virological micromethods. Acta microbiol. Acad. Scient. Hung. 3 191–202, 1955

25 Tan E Relationship of nuclear staining patterns with precipitating antibodies in systemic lupus erythematosus J Lab. & Clin. Med. 70 800–812, 1967

26 *Weller T H & Coons, A H* Fluorescent antibody studies with agents of varicella and herpes zoster propagated in vitro. Proc. Soc. Exp. Biol (N Y) 86 789–794 1954

tions and transcriptions, but are to a greater extent due to strandbreaks and fragmentations produced during the purification. Especially single stranded fringes at the ends of linear double helix fragments may be important in that they cause non specific reactions. The latter type of sugarbase exposure can be prevented if circular DNA molecules, usually from bacteria and vira, are used (2) At present, the former types of exposure are apparently not easily prevented. In all procedures utilizing isolated, supposedly native, DNA it is therefore necessary to control the specificity of the reactions by way of antisera with known specificity for native denatured and single stranded DNA, respectively Standard procedures for the preparation of DNA antigens and standard criteria for an evaluation of their quality are obviously needed and international recommendations in these respects seem to be in progress.

There was definitely no cross-reactivity between the DNA and ENA haemagglutination reaction. This is obvious from Tables 7 and 8 and it is further demonstrated in Fig 2 In addition the tables show the associations between these two haemagglutination reactions and the nuclear immunofluorescence pattern. The observed correlations are in agreement with the common concept that homogeneous and speckled nuclear patterns are produced by distinct antigen-antibody systems representing separate non cross-reacting specificity groups. The homogeneous group comprises nucleoprotein, d DNA, s-DNA and histone, probably also a number of unidentified substances. The incomplete correlations which are apparent from the tables, can be explained if it is assumed that a speckled fluorescence pattern may be concealed by a homogenous pattern or that a weak homogeneous pattern may be ignored owing to a contrast effect in the presence of a strong speckled reaction It may also occur that two or more speckled patterns are intermingled to cause a homogeneous appearance

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REFERENCES

1 *Aarden L. A de Groot E. R. & Feltkamp T E. W* Immunology of DNA III *Crithidia luciliae* a simple substrate for the determination of anti-ds-DNA with the immunofluorescence technique. Ann N Y Acad. Sci. *254* 505–515 1975

2 *Aarden L. A Lakmaker F de Groot E. R Swaak A J G & Feltkamp T E. W.*, Detection of antibodies to DNA by radioimmunoassay and immunofluorescence. In Feltkamp, T E W (Ed.) The significance of the determination of anti DNA and DNA/anti DNA complexes. Proc. 1 st Bertine Coperberg Conf. Scand J Rheum. Suppl. *11* 12–19 1975

3 *Allen R J L.* The estimation of phosphorous. Biochem. J *34* 858–865 1940

4 *Bergquist R* Immunofluorescence. Evaluation of techniques and reagents with special reference to methodological standardization. Thesis, Karolinska Institutet, Stockholm 1974 pp 21–24 30–39

5 *Boyden S V* The adsorption of proteins on erythrocytes treated with tannic acid and subsequent haemagglutination by anti-protein sera. J Exp Med. *93* 107–120 1951

6 *Burton K* A study of the conditions and and mechanism of the diphenyl amine reaction for the colorimetric estimation of desoxyribonucleic acid. Biochem. J *62* 315–323 1944

7 *Davidson J N* The Biochemistry of the nucleic acids (7th Ed) Chapman & Hall/Science Paperbacks 1972 Chapt. 9

8 *Espmark A Biberfeld G Fagraeus A Johnsson T Johnsson J & Magnusson B.* Virological findings during the smallpox outbreak in Stockholm in 1963 Acta Med. Scand. Suppl *464* 71–86 1966

9 *Fagraeus A Espmark A & Jonsson J* Mixed haemadsorption. A mixed antiglobulin reaction applied to antigens on a glass surface Preparation and evaluation of indicator cells Survey of present applications. Immunology *9* 161–175 1965

10 *Jonsson J* Symptomatology and diagnosis in connective tissue disease. Antibodies to extractable ribonucleoprotein in 123 patients re-

the disease-inducing activity of region 1–42 in rabbits

MATERIAL AND METHODS

Animals and sensitization. Random-bred albino rabbits weighing 2 2½ kg were obtained from Møllegaards Avlslab Ejby, Denmark. They were sensitized with a total amount of each peptide, as specified below. For this purpose, peptide solutions of appropriate concentration were emulsified with equal volumes of Freund's complete adjuvant (Difco Lab.). Each animal received 0.5 ml of the specified antigen emulsion in three footpads.

Animals were killed when showing unequivocal clinical signs of disease (hindleg paralysis, mostly in combination with incontinence) or about 35 days after sensitization. Brains and three specified parts of the spinal cords were removed and processed for histological examination, as previously described (Lindh & Bergstrand 1975). The levels thus examined were cranial, middle and caudal parts of hemispheres, midbrain, cerebellum with oblongata, cervical, thoracic, and lumbar spinal cord levels. Histological examination (on coded slides) was made by Dr. *B. Källén*.

Antigens. The peptides tested for encephalitogenicity were all derivatives of peptide 1–42 of bovine encephalitogenic protein. The procedures used for preparing and characterizing that peptide and peptides 1 19, 16–36, 12 23, and 32–41 have previously been described in detail (*Bergstrand* 1971, *Bergstrand & Källén* 1973), although numbering of amino acid residues has been changed to conform to the revised primary sequence of the protein (*Brostoff et al.* 1974).

The peptide fractions referred to as 1 12, and 37–42 were isolated by CM Sephadex chromatography and Sephadex G-50 superfine gel filtration from peptic digest of peptide 1–42 (produced by treating 125 mg 1 42 in 10 ml 0.25 M ammonium acetate buffer pH 3.5 with 4 mg pepsin (Sigma) for 4 hours at 37 C). Amino acid analyses of the purified peptides was performed as described elsewhere (*Bergstrand* 1971). Identification of peptides depended on these amino acid analyses and estimates of their molecular weights obtained by gel filtration as described elsewhere (*Bergstrand* 1971).

Peptide 1 19 was succinylated at the two lysine residues as follows (cf *Bergstrand & Källén* 1973 a). To 2.5 mg of peptide 1 19 in 5 ml distilled water was added 4 mg of succinic anhydride in small portions, the pH being maintained at 7–8 by the addition of 0.5 M NaOH. The reaction mixture was left for 30 min at room temperature and then lyophilized. According to the reaction with trinitrobenzene sulfonic acid (see *Habeeb* 1967) succinylation of the lysine residues was complete.

All peptides were sterilized by Millipore filtration and stored lyophilized in aliquots at −20 C until used. Calculation of doses is based on dry weights of lyophilized peptide materials.

RESULTS AND DISCUSSION

The present report describes further attempts to localize the encephalitogenic site(s) residing in region 1–42 of bovine encephalitogenic protein.

Table 1 gives the results of amino analyses of peptide fractions 1–12, and 37–42. Peptide 1–12 was free of detectable contamination the analyses suggest some degree of contamination for peptide 37–42. Amino acid analyses of other peptides agree with previously reported results (*Bergstrand* 1971 *Bergstrand & Källén* 1973 a, b)

TABLE 1 *Amino Acid Composition of Peptide Fractions 1–12 and 37–42*

	Peptide 1–12		Peptide 37 42	
Lys	1 97	(2)	0.20	
His	0.17		0 08	
Arg	2 17	(2)	0.79	(1)
Asx	0.17		1 13	(1)
Thr	0.13		0.28	
Ser	1.97	(2)	1 17	(1)
Glx	2 20	(2)	0 27	
Pro	0.90	(1)	0 05	
Gly	0 27		0 97	(1)
Ala	2.17	(2)	0 50	
Val	0 03		0 11	
Met	0.05		0.08	
Ile	0.09		0.15	
Leu	0.13		1.29	(1)
Tyr	0.97	(1)	0.1	
Phe	0 03		0 69	(1)

The figures are the molar ratios with reference to the estimated molecular weight. Figures in brackets are those to be expected according to the primary sequence (*Brostoff et al.* 1974)

Table 2 shows the results obtained at examination of the encephalitogenicity of the various peptides in rabbits. In a few cases the histological examination revealed almost no

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ENCEPHALITOGENIC ACTIVITY OF THE N-TERMINAL PART OF BOVINE MYELIN BASIC PROTEIN IN RABBITS

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Bergstrand, H. Encephalitogenic activity of the N terminal part of bovine myelin basic protein in rabbits. Acta path. microbiol. scand. Sect. C, 84: 442–446, 1976.

The disease-inducing activity of the N terminal part (residues 1–42) of bovine myelin basic protein in rabbits is localized to two regions. One encephalitogenic determinant probably incorporates at least one of the lysine residues (Nos. 4 and 11), the other is within residues 20–36. It is concluded that region 1–42 shows a comparably strong degree of encephalitogenicity for rabbits.

Key words: Bovine myelin basic protein; N terminal part; encephalitogenic activity; rabbits.

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Experimental allergic encephalomyelitis (EAE) is an autoimmune disease induced in animals by injections of central nervous tissue suitably incorporated in an adjuvant (for recent reviews, see *Kies* 1973, *Rauch & Einstein* 1974). The main encephalitogenic principle has been identified as the basic protein of myelin. Recent studies in several laboratories have led to an extensive characterization of this protein. It has been shown that the disease-inducing activity of the protein can be traced to a number of defined encephalitogenic determinants. The activity of most of these determinants seems to depend on the origin of the antigen and the species employed for testing it (for reviews, see *Kies* 1973, *Rauch & Einstein* 1974, *Bergstrand* 1976).

Peptide 1–42 of bovine encephalitogenic protein or parts of it has previously been shown to express a slight to moderate degree of disease inducing activity in monkeys, guinea pigs, and rats (for references, see *Bergstrand* 1976). Peptide 1–19 has been ascribed a moderate activity in rabbits (*Shapira et al.* 1971) and we have previously reported that peptide 1–42 of bovine encephalitogenic protein at a dose level of 16 nmoles (75 µg) induces EAE in practically all the injected rabbits (see *Bergstrand & Kallén* 1973 b, *Berg & Bergstrand* 1974). Preliminary experiments with peptide 20–42 indicated that this fragment retained at least part of the activity (*Berg & Bergstrand* 1974). These data seemed to place the specific encephalitogenic activity of region 1–42 when studied in the rabbit in an order of magnitude close to that of region 43–88 which is thought to incorporate the major encephalitogenic site of the bovine myelin basic protein for this species (*Kibler & Shapira* 1968, *Shapira et al.* 1971). Thus 50 µg of region 43–88 (10 nmoles) induce maximal EAE in rabbits (*Kibler & Shapira* 1968).

We presently report on extended studies of

the disease-inducing activity of region 1–42 in rabbits.

MATERIAL AND METHODS

Animals and immunization. Random-bred albino rabbits weighing 2–2½ kg were obtained from Hallegaards Avlslab, Skensved, Denmark. They were immunized with total amount of each peptide, as specified below. For this purpose, peptide solutions of appropriate concentration were emulsified with equal volumes of Freund' complete adjuvant (Difco Lab.). Each animal received 0.5 ml of the specified antigen emulsion in three footpads.

Animals were killed when showing unequivocal clinical signs of disease (hindleg paralysis, mostly in combination with incontinence) or about 35 days after immunization. Brains and three specified parts of the spinal cords were removed and processed for histological examination, as previously described (*Lindh & Bergstrand* 1975). The levels thus examined were cranial, middle and caudal parts of hemispheres, midbrain, cerebellum with oblongata, cervical, thoracic, and lumbar spinal cord levels. Histological examination (on coded slides) was made by Dr. *B. Källén.*

Antigens. The peptides tested for encephalitogenicity were all derivatives of peptide 1–42 of bovine encephalitogenic protein. The procedures used for preparing and characterizing that peptide and peptides 1–19, 16–36, 12–23 and 32–41 have previously been described in detail (*Bergstrand* 1971, *Bergstrand & Källén* 1973 a), although numbering of amino acid residues has been changed to conform to the revised primary sequence of the protein (*Brostoff et al.* 1974).

The peptide fractions referred to as 1–12, and 37–42 were isolated by CM-Sephadex chromatography and Sephadex G-50 superfine gel filtration from peptic digest of peptide 1–42 (produced by treating 125 mg 1–42 in 10 ml 0.25 M ammonium acetate buffer pH 3.5 with 4 mg pepsin (Sigma) for 4 hours at 37 C). Amino acid analysis of the purified peptides was performed as described elsewhere (*Bergstrand* 1971). Identification of peptides depended on these amino acid analyses and estimates of their molecular weights obtained by gel filtration as described elsewhere (*Bergstrand* 1971).

Peptide 1–19 was succinylated at the two lysine residues as follows (cf. *Bergstrand & Källén* 1973): T 2.5 mg of peptide 1–19 in 5 ml distilled water was added 4 mg of succinic anhydride in small portions, the pH being maintained at 7.5–8 by the addition of 0.5 M NaOH. The reaction mixture was left for 30 min at room temperature and then lyophilized. According to the reaction with trinitrobenzene sulfonic acid (see *Habeeb* 1967) succinylation of the lysine residues was complete.

All peptides were sterilized by Millipore filtration and stored lyophilized in aliquots at –20 C until used. Calculation of doses is based on dry weights of lyophilized peptide materials.

RESULTS AND DISCUSSION

The present report describes further attempts to localize the encephalitogenic site(s) residing in region 1–42 of bovine encephalitogenic protein.

Table 1 gives the results of amino analyses of peptide fractions 1–12, and 37–42. Peptide 1–12 was free of detectable contamination the analyses suggest some degree of contamination for peptide 37–42. Amino acid analyses of other peptides agre with previously reported results (*Bergstrand* 1971 *Bergstrand & Källén* 1973 a, b).

TABLE 1 *Amino Acid Composition of Peptide Fractions 1–12 and 37–42*

	Peptide			
	1–12		37–42	
Lys	1.97	(2)	0.20	
His	0 17		0 08	
Arg	2 17	(2)	0.79	(1)
Asx	0 17		1 13	(1)
Thr	0 13		0 28	
Ser	1.97	(2)	1 17	(1)
Glx	2.20	(2)	0 27	
Pro	0.90	(1)	0 03	
Gly	0.27		0 97	(1)
Ala	2.17	(2)	0.30	
Val	0 03		0 11	
Met	0.03		0.08	
Ile	0 09		0 15	
Leu	0 13		1 29	(1)
Tyr	0 97	(1)	0.12	
Phe	0 03		0 69	(1)

The figures are the molar ratios with reference to the estimated molecular weight. Figures in brackets are those to be expected according to the primary sequence (*Brostoff et al.* 1974).

Table 2 shows the results obtained at examination of the encephalitogenicity of the various peptides in rabbits. In a few cases the histological examination revealed almost no

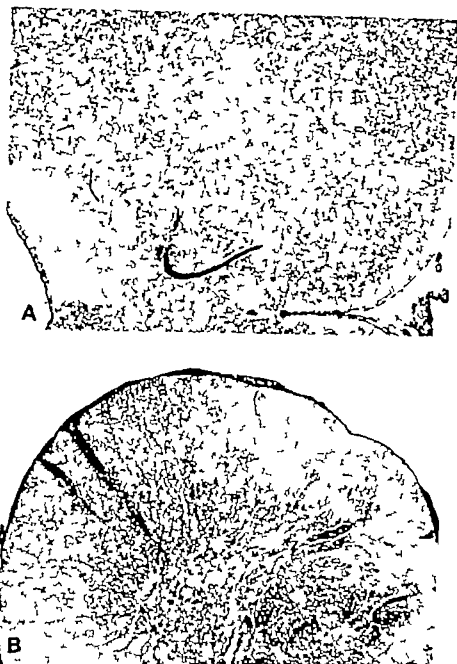


Fig 1 Cross-sections through the brainstem (A) and caudal part of the spinal cord (B) from a rabbit injected with peptide 1–19 Note very slight pathological changes in A consisting of a small vascular cuff but heavy meningo-encephal tis n B with thick ascular cuffs and cellular infiltration. Magn. 30 ×

lesions in brain tissue whereas the lower part of the spinal cord was heavily affected by cellular infiltration (Fig 1)

The results in Table 2 indicate that region 1–42 encompasses at least two sequences with a comparably high disease inducing activity One seems to reside in the N terminal part because (i) peptide 1–19 is considered active ((ii) blocking lysine residues 4 and 11 of peptide 1–19 by succinylation abolished the activity and (iii) peptide 1–12 but not peptide 12 23 seems capable of inducing EAE These data suggest that at least one of the two lysine residues forms part of an encephalitogenic determinant. The tentative location of this determinant seems close to that reported

for a disease-inducing sequence of the human myelin basic protein active in guinea pigs (*Burton et al.* 1972).

TABLE 2. *Encephalitogenic Activity of Various Peptides Derived from Peptide 1–42 of Bovine Encephalitogenic Protein in Rabbits. Animals Characterized as Clinically Affected Showed Unequivocal Paresis of Hind Legs*

Peptide	Dose	No. of animals affected	
		Clinically	Histologically
1–42*	16 nmol	13/14	14/14
1–19	36	4/4	2/4‡
1–19, acetylated	215	0/4	1/4†
1–12	34	1/4	1/4
	225	3/4	3/4
17–23	38	0/4	0/4
	170	0/4	1/4
16–36	33	3/4	3/4
32–41	36	0/4	0/4
	220	0/4	0/4
37–42	225	0/4	0/4

* Data from *Bergstrand & Källén* 1973 b, and *Berg & Bergstrand* 1974.
‡ Only brains, not spinal cords, were histologically examined.
† Histological signs of positive animal mild.

A second encephalitogenic site seems to constitute some part of region 20–36. Thus, peptide 20–42 was previously shown to be active (*Berg & Bergstrand* 1974) and we presently found low doses of peptide 16–36 capable of inducing disease. Attempts at a further localization of this encephalitogenic determinant showed that neither peptide 32–41 nor peptide fraction 37–42 was active. Taken together, the data suggest that the C-terminal part is not responsible for the disease inducing activity of region 20–42.

Although we have not encountered the difficulties reported by others (*Eylar et al.* 1974) in inducing EAE in rabbits, it is clear that this species is not quite suitable for performing adequate bioassays of encephalitogenicity (see *Kies* 1973). Thus, we realize that the present examination, performed with rather small groups of animals, can result only in a tentative localization of the encephalitogenic determinants. A more stringent examination of their location and specific activity awaits synthesis of suitable derivatives of peptide 1–42. Provisionally, our combined results indicate that peptide 1–42 contributes substantially to the activity of the encephalitogenic protein in the rabbit. Moreover, they extend the number of sites claimed to induce EAE in this species to at least six (incorporated within regions 1–19, 20–36, 43–67 and/or 65–74, 114–122, 133–149 and 153–169; for references see *Bergstrand* 1976).

The cost of the present examination was defrayed with grants from the *Swedish Medical Research Council* (B75-16X-3920) and research funds of the *Medical Faculty, University of Lund*.
Amino acid analyses were kindly performed by Dr. *L. B. Sjöberg*, Astra Nutrition.

REFERENCES

Burton, M. A., McPherson, T. A., Lemieux, R. U. & Bain, G. O.: Experimental allergic encephalomyelitis (E.A.E.). Synthesis of the 7–11, 11–20 and 12–20 peptides of human encephalitogenic protein and inhibition of histological E.A.E. by the 12–20 peptide. Can. J. Biochem. *50*: 689–696, 1972.
Berg, O. & Bergstrand, H.: Antibodies with a cytotoxic effect in sera from rabbits injected with peptide 1–43 of the bovine encephalitogenic protein. Neurobiology *4*: 191–196, 1974.
Bergstrand, H.: Isolation and partial characterization of some proteolytically and chemically derived fragments of bovine encephalitogenic protein. Eur. J. Biochem. *21*: 116–124, 1971.
Bergstrand, H.: Immunochemistry of encephalitogenic protein. In: *Atassi, M. Z.* (Ed.): Immunochemistry of proteins. Plenum Press, N.Y. 1976 (In press).
Bergstrand, H. & Källén, B.: Antigenic determinants in the N-terminal part of bovine encephalitogenic protein studied with the macrophage migration inhibition assay in guinea pigs. Evidence for an immunological reactivity of peptide 1–43 when tested on cells from animals injected with Freund's complete adjuvant. Immunochem. *10*: 229–238, 1973 a.
Bergstrand, H. & Källén, B.: Antigenic determinants on bovine encephalitogenic protein. Studies in rabbits with derivatives of fragment 1–43 and the lymph node cell transformation test. Neurobiology *3*: 246–255, 1973 b.

Brostoff S W Reuter W Hichens M & Eylar E H Specific cleavage of the A1 protein from myelin with Cathepsin D J Biol Chem. *249* 559–567 1974

Eylar E. H Kniskern P J & Jackson J J Myelin basic proteins Methods in Enzymology *XXXII* (Part B) 323–341 1974

Habeeb A F S A Preparation of enzymatically active water-insoluble derivatives of trypsin. Arch. Biochem. Biophys. *119* 264–268, 1967

Lindh J & Bergstrand H Disease-inducing activity of different parts of bovine encephalitogenic protein in guinea pigs. Neurobiology *5* 137–150 1975

Kibler R F & Shapira R. Isolation and properties of an encephalitogenic protein from bovine, rabbit, and human central nervous system tissue. J Biol Chem. *243* 281–286 1968.

Ases M W Experimental allergic encephalomyelitis. In *Gaull, G E* (Ed.) Biology in Brain Dysfunction, Vol. 2, Plenum Press, N.Y 1973 p 185–224

Rauch H C & Einstein E. R Specific brain proteins A biochemical and immunological review In *Ehrenpreis S* and *Kopin I J* (Eds.) Rev Neurosciences. Raven Press, N Y 1974 p 283–343

Shapira R Chou F C.-H McKneally S., Urban E. & Kibler R F Biological activity and synthesis of an encephalitogenic determinant. Science *173* 736–739 1971

Acta path. microbiol. scand. Sect. C, 84 447–454 1976

THE CYTOTOXICITY OF SPECIFICALLY SENSITIZED LYMPHOCYTES FROM MOUSE STRAINS OF VARYING H 2 SPECIFICITIES ON LCM VIRUS-INFECTED L CELLS

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Marker O & Andersen, G T The cytotoxicity of specifically sensitized lymphocytes from mouse strains of varying H 2 specificities on LCM virus-infected L cells. Acta path. microbiol. scand Sect C, 84 447–454 1976

During an LCM virus infection in mice, cytotoxic T lymphocytes appear to be directed against target cells infected with the virus This cytotoxic reaction is restricted by the H 2 histocompatibility between effector and target cells To analyse this restriction, the cytotoxic effect of in vivo sensitized spleen cells from mice of various H 2 specificities on LCM virus-infected L cells (H 2^k) was studied in vitro When spleen cells from strains identical with C3H (H-2^k) and L cells at the K as well as at the D locus were used as effector cells, the degree of lysis was of the same order as that obtained by C3H effector cells, whereas strains different at both loci did not respond significantly The A/J and B10A strains, both identical with the L cells at the K locus but different at the D locus, gave low or moderate responses. C3H H 2^o lymphocytes, identical with the targets at the D but not at the K locus, showed high degrees of cytotoxicity although the level obtained by strains histocompatible with L cells at both loci was never reached. Effector cells from the F1 hybrids C3H/HeJ × BALB/c and C3H/HeJ × DBA both responded poorly in spite of the fact that one of their haplotypes carries the same marker loci as the L cells This finding is in contrast with that obtained in other laboratories where the LCM virus system and other systems were studied. A point is made of the design and the evaluation of the experiments.

Key words H-2 histocompatibility lymphocytic choriomeningitis virus T-lymphocyte mediated cytotoxicity

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Over the last two years several articles have appeared which emphasize the necessity of H 2 histocompatibility between effector cells and target cells in the specific T cell-mediated lysis of chemically modified (7 9 15 16) pox virus-infected (1 11) and LCM virus-infected (4 5 6, 18 20 21) target cells.

Where the LCM virus studies are concerned, the data obtained arise from *in vivo* as well as from *in vitro* studies. They seem to

demonstrate not only that total identity at the H 2 gene complex is essential to maximal cell lysis of LCM virus-infected target cells but also that compatibility at either the H 2K or at the H 2D locus is sufficient (4) Likewise, these data indicate that sensitized lymphocytes from F1 hybrids are able to lyse infected target cells H 2 compatible with one of the parental strains as efficiently as effector cells from the parental strain itself (20) The results obtained by chemically modified surface antigens are to a certain degree analogous. In some systems, however, compatibility at the D locus alone is not sufficient for full lysis (7 15) and some data may indicate that H 2K similarity results in submaximal responses (15)

In an attempt to confirm some of these results, we have analysed the cytotoxic effect of *in vivo* sensitized spleen cells from different mouse strains on LCM virus-infected L cells.

As regards the strains used we found that identity at both the K and the D locus of the H 2 complex ensures 100 per cent lysis while on the other hand lack of identity at the K as well as at the D locus makes lysis impossible.

In other respects however our results differ from the original findings of other investigators (4 20) As opposed to the latter we found that identity between effector cells and target cells either at the H 2K or at the H 2D locus is not sufficient for full lysis to occur and in our experiments lymphocytes from the immunized F1 hybrids C3H/HeJ × BALB/c and C3H/HeJ × DBA showed very low degrees of cytotoxicity for virus-infected L cells.

The purpose of the present study is partly to give an account of our methods by which the cytotoxicity of various populations of sensitized lymphocytes is compared partly—on the evidence of our results—to raise the question whether the early results obtained in the LCM virus system could have turned out differently if another method of evaluation had been employed.

MATERIALS AND METHODS

Mice

Inbred AKR/J/Ssc (H 2k) CBA/J/Ssc (H 2k) C3H/HeJ/Ssc (H 2k) and C3H/Ssc 1 (H-2k) mice were obtained from Statens Seruminstitut, Copenhagen, where the C3H/Ssc 1 mouse had been bred for about 20 years.

The genotypes A/J (H 2a) B 10.A (H 2a) C3H *H* 2o/ScSn (H 2o) DBA/2J (H 2d) C57Bl/6J (H 2b) BALB/c (H 2d) and A.CA (H 2f) were bred in this laboratory

The hybrids used were C3H/HeJ × DBA/2 (H 2k × H 2d) and C3H/HeJ × BALB/c (H 2k × H 2d) These animals were purchased from Laboratory Animals Breeding and Research Centre, DK-8680 Ry

Lymphocytic Choriomeningitis (LCM) Virus

The Traub strain was used as described previously (14)

Cytotoxic Test

The 51Cr release test used was the Brunner assay (2) and a slight modification of that already described (13)

Target cells L cells were infected and labelled as described in a previous paper (13) After five washings, the cells were resuspended in MEM and distributed into Petri dishes, each receiving 2 6 × 10⁴ cells. The dishes were kept at 37 C for 2 h in a humid atmosphere containing 3.5 per cent CO_2 to allow the cells to adhere to the bottom. The efficiency of the infection was checked intermittently by the immunofluorescence technique and found to be 95–100 per cent. The 51Cr release from uninfected L cells was tested in the same experiments and was always found to be the same as that from infected cells with medium alone.

Lymphocyte suspensions. Five mice of each strain were used in all experiments. The mice were infected 8 or 9 days prior to the experiment (12, 13) On the day of the experiment, the spleen cells were harvested from all mice and single-cell suspensions of spleen cells were prepared as described in a previous paper (13) Spleen cells were added to target cells in the following ratios 25 1 12.5 1 and 6 25 1 C3H/Ssc 1 used in all experiments as a positive reference, was tested additionally by the ratio 3 125 1 in 11 out of 18 experiments. Five replicate dishes were tested with each ratio.

Spontaneous Cr release Preliminary experiments had shown that there was no significant difference between the Cr release from cultures of target cells alone and from cultures with normal spleen cells from any of the mouse strains used. In all subsequent experiments the spontaneous release was therefore measured in cultures of target

cells and normal C3H/Sec 1 lymphocytes by the ratio 25:1. With normal lymphocytes, the release never constituted more than 30–35 per cent of the total.

Determination of Cr release The reaction mixtures were incubated for 18 h at 37 C in humid atmosphere containing 3.5 per cent CO_2. Samples from each Petri dish were then transferred to small glass tubes and centrifuged at 400 *G*. Subsequently 1.00 ml of the supernatant was transferred to plastic counting vials and the amount of radio-isotope was determined in a gamma counter.

Calculation and Evaluation

The target cell destruction was expressed as a cytotoxic index (CI) reflecting the ratio between the actual amounts of Cr released and the total amounts incorporated. The amounts of radio-isotope were measured as counts per minute, and the following fraction was used for the calculation (2)

$$CI = \frac{(\text{Cr release in the presence of immune lymphoid cells}) - (\text{Cr release in the presence of normal lymphoid cells})}{(\text{Total Cr incorporated}) - (\text{Cr release in the presence of normal lymphocytes})} \times 100$$

When the CI of all ratios and strains had been calculated, curves were traced for each strain by plotting the CI for each lymphocyte target cell ratio versus log ratio on common graph paper. Fig. 1 depicts the results of one experiment. From the curves of each experiment, the ratio of lymphocytes to target cells necessary to obtain 50 per cent lysis was determined graphically. The number of lymphoid cells corresponding to this ratio has been defined arbitrarily as one lytic unit (LU-50). The number of LU 50s present in 10^6 lymphocytes was then calculated and employed for later evaluation (3). The 50 per cent level was chosen in preference to the 33 per cent level suggested by *Cerottini* & *Brunner* (3) as, in our experiments, this provided greater certainty of hitting the linear (logarithmic) sequence of the curve.

The curves traced from low responses (Fig. 1) had a very slight slope. In these cases, the number of LU 50s was determined from the intersecting point between a line parallel to the linear segment of the C3H/Sec 1 curve through the point indicating the CI with ratio 25 and the line indicating 50 per cent lysis. The results of these low responses are stated as < the calculated value.

RESULTS

From the main diagram of Fig. 1 it is evident that for the strains C3H/Sec 1 and C3H H 2 /ScSn, the cytotoxicity is the same with ratio 25. The remaining part of the curves, however, reveals appreciable differences between the cytotoxic responses of the two strains. Furthermore, the linear (logarithmic) parts of the two curves are seen to run in parallel.

Table 1 summarizes the results of 18 experiments. Usually each experiment comprised 3 or 4 strains, and C3H/Sec 1 was included in each experiment as a positive control.

The number of LU 50s/10^6 spleen cells was, for each strain, expressed in percentages of that obtained with C3H/Sec 1. The figures in Table 1 state these percentage results from the single experiments.

It appears from the table that all strains compatible with L cells at both the K and the D locus develop spleen cell populations with the same (or higher) cytotoxic activity as that of the C3H/Sec 1 strain. Likewise, it is obvious that spleen cells from the strains which do not share H 2 antigenic specificity with L cells either at the K or at the D locus are not cytotoxic for this type of cells infected with LCM virus.

Mice from A and B 10.A strains, which both show H 2 compatibility with L cells only at the K locus, have developed lymphocytes with different degrees of cytotoxic activity. Thus, in 3 cases A/J mice gave responses that were too low to allow a correct comparison with C3H/Sec 1, whereas in 1 case the response constituted 12.5 per cent of the C3H/Sec response. B 10.A, however presented intermediary degrees of cytotoxicity, the mean being 30 per cent ± 6.7 (standard error of the mean) of the C3H/Sec 1 response.

C3H H 2 /ScSn mice which are H 2 compatible with L cells only at the D locus gave high cytotoxic responses with a mean of 72 per cent ± 5.8. It should be noted, however, that the response never became

demonstrate not only that total identity at the H 2 gene complex is essential to maximal cell lysis of LCM virus-infected target cells, but also that compatibility at either the H 2K or at the H 2D locus is sufficient (4) Likewise, these data indicate that sensitized lymphocytes from F1 hybrids are able to lyse infected target cells H 2 compatible with one of the parental strains as efficiently as effector cells from the parental strain itself (20) The results obtained by chemically modified surface antigens are to a certain degree analogous. In some systems, however, compatibility at the D locus alone is not sufficient for full lysis (7 15) and some data may indicate that H 2K similarity results in submaximal responses (15)

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The genotypes A/J (H 2) B 10.A (H-2) C3H *H 2* /ScSn (H $2^•$) DBA/2J (H 2^d) C57Bl/6J (H 2^b) BALB/c (H 2^d) and A.CA (H 2^f) were bred in this laboratory

The hybrids used were C3H/HeJ × DBA/2 (H 2^k × H 2^d) and C3H/HeJ × BALB/c (H 2^k × H 2^d) These animals were purchased from Laboratory Animals Breeding and Research Centre, DK-8680 Ry

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TABLE 1 *Number of LU 50/10⁶ Spleen Cells from Different Strains of Mice in Percentage of the C3H/Sec 1 Response*

Mouse strain (effector cells)	H-2 alleles K	H-2 alleles D	L cells (target cells) K	L cells (target cells) D	Cytotoxicity in percentages of the C3H/Sec 1 response						
C3H/Sec 1	k	k	k	k	100	100	100	100	100	100	100
C3H/HeJ	k	k	k	k	>100	>100	>100	100	>100	>100*	
CBA/J	k	k	k	k	>100	>100	>100	>100			
AKR/J	k	k	k	k	>100	>100*					
A/J	k	d	k	k	< 11	< 3	13	< 11			
B10.A	k	d	k	k	36	53	18	17	24‡		
[illegible]	d	k	k	k	89	69	63	67†			
DBA/2J	d	d	k	k	0	0					
C57BL/6J	b	b	k	k	< 9	< 14	< 10				
BALB/c	d	d	k	k	< 16	0					
CA	f	f	k	k	0	< 10					
C3H/HeJ x BALB/c	k d	k d	k	k	< 14	< 12	49	< 19	< 35	< 31	
C3H/HeJ x DBA	k d	k d	k	k	< 2	< 24	< 9	< 18	< 16	< 29	< 21

* The actual percentages calculated for these strains in each experiment have varied between 130 per cent and 500 per cent. They have not been included in the table, however, since the very high percentages do not correlate with similarly high degrees of cytotoxicity. They are to a large extent a consequence of the fact that the graphical depiction of the number of LU-50/10⁶ lymphocytes as a function of the log. ratio at 50 per cent lysis is an equilateral hyperbola having a very steep slope in this area.
‡ 29.6 ± 6.7 (mean ± s.e.m.)
† 72.1 ± 5.8 (mean ± s.e.m.)

cases, the curves may correspond to the lower end of the S-shape and therefore have a very slight slope. Because the way in which we evaluate such results involves an extrapolation from the actual findings (Materials and Methods, Fig. 1) the calculated value, which is used as the upper limit of such results, is always the maximal one obtainable, and therefore in nearly all cases obviously much higher than the real level of the responses concerned.

Furthermore we have found that the maximal lysis obtainable in a single experiment can vary considerably from experiment to experiment. This corresponds graphically to different levels of the upper flattening of the S-shaped curve. We have thus found that maximal lysis in five consecutive experiments amounted to 83 per cent, 96 per cent, 74 per cent, 82 per cent and 62 per cent.

One set of explanations of this might be found in the target cells, the phenomenon being due to minor variations in the fragility of the L cells in tissue culture, the infection of these cells and thereby the expression of LCM virus specific antigens on the cell surface.

In an attempt at reducing the uncertainty caused by the target cells, we included in each experiment a known reference strain of high cytotoxicity in the system used, and the cytotoxic capacity of lymphocytes from other strains was calculated in proportion to this reference. The fact that the relative quantity of the cytotoxic response of different strains has remained relatively constant from experiment to experiment (Table 1) was considered to be in support of this practice.

The different responses achieved with various strains, as described here, should thus be due to differences in the cytotoxic capacity of the corresponding effector cell preparations. These differences are interpreted in this paper as being dependent on the H-2 gene

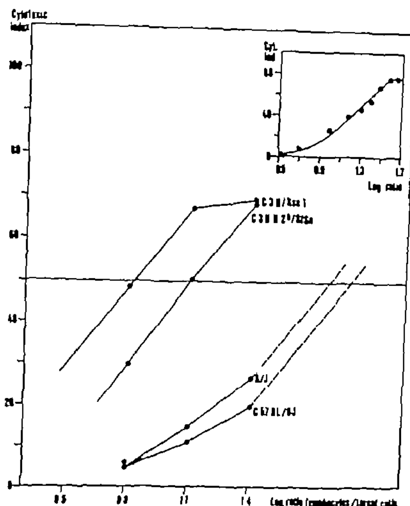


Fig. 1. Log ratio lymphocytes/target cells plotted against cytotoxic indices from a single experiment including four different strains of mice. Insertion: the course of cytotoxicity obtained with increasing numbers of sensitized lymphocytes and a constant number of target cells.

as high as that of the C3H/Sse 1 strain.

The F1 hybrids C3H × BALB/c and C3H × DBA gave extremely low responses in all experiments except one.

DISCUSSION

The curve depicting the results of a cytotoxic test of an increasing amount of sensitized lymphocytes and a constant number of target cells has a characteristic S-shape presenting an upper and lower flattening. This has been demonstrated by several investigators (3, 8, 17) and has also been observed by us (Fig. 1, insertion, unpublished results). For this reason the comparison between the cytotoxicity of different populations of lymphocytes must be made on the linear segment of the curve. If the comparison is made on the basis of the results achieved with a single ratio it is possible to obtain responses of the same magnitude for populations which, in fact, show different degrees of cytotoxicity as the indices obtained can be situated at one or the other of the two deflections of the curve. The only way in which to ensure that the linear segment of the curve is used for the evaluation is, in all experiments, to test the lymphocytes by three or more appropriate ratios in relation to the target cells.

The basic mechanism and the kinetics involved in different T cells killing of the same target cell carrying the sensitizing antigen on the surface must be expected to be the same, and it should therefore be demanded that the linear segments of the curves obtained in one and the same experiment have the same slope.

However, the very low responses still cause a problem in the evaluation since, in these

TABLE 1 *Number of LU 50s/10⁸ Spleen Cells from Different Strains of Mice in Percentage f the C3H/Sse 1 Response*

Mouse strain (effector cells)	H 2 alleles K	H 2 alleles D	L cells (target cells) K	L cells (target cells) D	Cytotoxicity in percentages of the C3H/Sec 1 response						
H/Sec 1	k	k	k	k	100	100	100	100	100	100	100
E/HeJ	k	k	k	k	*>100*	*>100*	*>100*	*100*	*>100*	*>100**	
R/J	k	k	k	k	>100	>100	>100	>100*			
K/J	k	k	k	k	>100	>100*					
J	k	d	k	k	< 11	< 3	13	< 11			
ILA	k	d	k	k	36	33	18	17	24‡		
3H *H-2ᵈ*	*d*	*k*	*k*	*k*	89	69	63	67†			
IA/2J	d	d	k	k	0	0					
57BL/6J	b	b	k	k	< 9	< 14	< 10				
ULB/c	d	d	k	k	< 16	0					
CA	f	f	k	k	0	< 10					
3E/HeJ x BALB/	k d	k d	k	k	< 34	< 12	49	< 19	< 35	< 31	
3H/HeJ x DBA	k d	k d	k	k	< 2	< 24	< 9	< 18	< 16	< 29	< 21

The actual percentages calculated *for these strains in* each experiment have aried between 150 per cent and 500 per cent. They have not been included in the table, however since the ery high percentages do not correlate with similarly high degrees of cytotoxicity They are to large extent consequence of the fact that the graphical depiction of the number of LU-50/10⁸ lymphocytes as a function of the log ratio at 50 per cent lysis is an equilateral hyperbola having ery steep slope in this area.

‡ 23.6 ± 6.7 (mean ± s.e.m.)

† 72.1 ± 5.8 (mean ± s.e.m.)

cases, the curves may correspond to the lower end of the S-shape and therefore have a very slight slope. Because the way in which we evaluate such results involves an extrapolation from the actual findings (Materials and Methods, Fig. 1) the calculated value, which is used as the upper limit of such results, is always the maximal one obtainable, and therefore in nearly all cases obviously much higher than the real level of the responses concerned.

Furthermore, we have found that the maximal lysis *obtainable* in a single experiment can vary considerably from experiment to experiment. This corresponds *graphically* to different levels of the upper flattening of the S-shaped curve We have thus found *that* maximal lysis in five consecutive experiments amounted to 83 per cent, 96 per cent, 74 per cent, 82 per cent and 62 per cent.

One set of explanations of this might be *found* in the target cells, the phenomenon being due to minor variations in the fragility of the *L* cells in tissue culture, the infection of these cells and thereby the expression of *LCM* virus specific antigens on the cell surface.

In an *attempt* at *reducing* the uncertainty caused by the target cells, we included in each experiment a *known* reference strain of high cytotoxicity in the system used, and the cytotoxic capacity of *lymphocytes from* other strains was calculated in proportion to this reference. The fact that the *relative* quantity of the cytotoxic response of different strains has remained relatively constant from experiment to experiment (Table 1) was considered to be in support of this practice.

The different responses achieved with various strains, as described here, should thus be due to differences in the cytotoxic capacity of the corresponding effector cell preparations. These *differences* are interpreted in this paper as being dependent on the H 2 gene

complex. It cannot be ruled out however, that the disparity in cytotoxicity found in lymphocytes from various mouse strains could be due to immunogenetic factors or pathogenetic conditions characteristic of each strain. Such differences may veil or hide an H 2 linked variation On the other hand, it would seem unlikely that such factors should play a major role for obtaining the results presented here since H 2 dependency of the cytotoxicity is obvious from Table 1

These factors can be considered only in experiments where effector cells from each mouse strain can be tested using infected macrophages from the same strain as targets (19)

In the *in vitro* experiments in the LCM virus system carried out by *Zinkernagel & Doherty* (20) the variations in effector cell capacity based on immunogenetic or pathogenetic differences may have been taken into account by using target cells totally syngeneic with the effector cells whereby differences due to the H 2 complex are eliminated. To our knowledge however they have not made sufficient allowance for the S-shape of the cytotoxicity curve or for the variation in the sensitivity of the target cells. In our experiments these latter uncertain factors have been taken into consideration as far as possible

As mentioned we have in some respects been able to confirm the findings obtained by the above authors whereas in other respects our results differ from theirs.

It seems clearly demonstrated in our system that similarity at the K or at the D locus alone is not sufficient for full lysis to occur In most systems similarity at the K locus alone is sufficient (7 15 16 20) while histocompatibility at the D locus alone is sufficient in some systems (9 20) but not in others (7 15)

Our results could easily be explained. Half the effector cells educated in A/J and B 10 A would be expected to react primarily with the products of the d allele of the D locus, and as this antigen is not present on the targets the cytotoxicity should be less than 100 per cent. In fact the sum of the means of B 10.A and C3H $H\ 2^{o}$ is strikingly close to 100 per cent.

It is also of interest to note that the results obtained in our laboratory fit in with the intimacy theory proposed by *Doherty & Zinkernagel* (5) better than those of these authors.

The great difference between the results obtained in the case of the B 10.A and the A/J strains which both are identical with L cells at the K locus, but not at the D locus, may indicate that factors other than different degrees of identity at the H 2 gene complex play a role for the cytotoxic efficiency in this case. The nature of such factors is unknown but immunogenetic and pathogenetic qualities might be of importance.

The difference in importance of partial identity at the K or at the D locus alone did not manifest itself unambiguously in our results. Important in this respect is that only one strain sharing D locus characteristics with the L cell has been available. One possible explanation of the difference between the response of the A/J and B10.A strains on one side and that of the C3H H 2^{o} strain on the other could be, however that the antigenic status of the L cells used in this study expresses D-antigens stronger than K-antigens as compared with normal H 2^{k} cells. On the other hand from this institute there is recent evidence that these L cells do express antigens determined by genes at the K and the D locus with the same strength (10)

In the light of the genetic similarity of F1 hybrids and the parental strain intermediate responses from lymphocytes from the F1 strain to virus-infected target cells H 2 compatible with one parental strain might be expected The very low responses obtained in the present investigation are difficult to interpret and need confirmation.

The discrepancies mentioned could also be attributable to the different L cells which have been employed in different laboratories Such strains of tissue culture cells might have developed neoantigens on the cell surface. However we have not found it likely that such neo-antigens should have altered the

expression of the H 2 allele of the L cells since, using several mouse strains, it has been possible to confirm the demonstrated importance of full, or entirely failing, identity at the H-2 locus.

The fact that we have examined in detail only one system prevents conclusive interpretations of our results, but experiments with macrophages of different H 2 specificities used as target cells (18) as well as attempts at confirming our results in vivo are in progress in our laboratory and preliminary in vivo results fit extremely well with the in vitro results of the present study

The authors wish to express their thanks to Professor *Erna Möller* Karolinska Institutet, Stockholm, for valuable criticism of the manuscript. We also thank Dr *Peer Nebert Jørgensen* of this institute for having provided us with mice of several inbred mouse strains and for many fruitful discussions.

REFERENCES

1 *Blanden, R. V. Doherty P C Dunlop M B. C Gardner I D Zinkernagel, R. M & David C S* Genes required for T cell-mediated cytotoxicity against virus-infected target cells are in the K or D regions of the H 2 gene complex *Nature 254* 269–270 1975.

2 *Brunner K T Mauel, J., Cerottini J C & Chapuis B* A quantitative assay of the lytic action of immune lymphoid cells on [51]Cr labeled allogeneic target cells in vitro inhibition by isoantibody and by drugs Immunology *14* 181 195, 1968

3 *Cerottini, J.-C & Brunner T* In Vitro assay of target cell lysis by sensitized lymphocytes I Bloom, B R & Glade P R (Ed) In vitro methods in cell-mediated immunity Academic Press, New York & London, 1971 p 369–373

4 *Doherty P C & Zinkernagel, R. M* H 2 compatibility is required for T cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. J Exp Med. *141* 502 507 1975

5 *Doherty P C & Zinkernagel R M* A biological role for the major histocompatibility antigens Lancet June 28, p. 1406–1408, 1975.

6 *Doherty P C & Zinkernagel, R M* Enhanced immunological surveillance in mice heterozygous at the H-2 gene complex. Nature 256 50–52, 1975

7 *Forman J.* The role of the H-2 histocompatibility complex in determining the specificity of cytotoxic effector cells sensitized against syngeneic trinitrophenyl-modified targets. J Exp. Med. *142* 403–418, 1975

8. *Gardner I., Bowern Narella A. & Blanden R. V.* Cell-mediated cytotoxicity against ectromelia virus-infected target cells. II Identification of effector cells and analysis of mechanism. Eur J Immunol. *4* 68–72, 1974

9 *Gordon R. D., Simpson E. & Samelson L. E.* In vitro cell-mediated immune responses to the male specific (H Y) antigen in mice. J Exp. Med. *142* 1108–1120, 1975.

10. *Jørgensen, P N.* Incompatibility at irrelevant H-2 specificities augments in vivo stimulation of alloaggressive cells. Scand. J Immunol. *4* 373–382, 1975.

11 *Koszinowski U & Thomssen R.* Target cell-dependent T cell-mediated lysis of vaccinia virus-infected cells. Eur J Immunol. *5* 245–251 1975

12. *Marker O & Volkert M* In vitro measurement of the time course of cellular immunity to LCM virus in mice. In Lehmann-Grube, F (Ed.) Lymphocytic Choriomeningitis Virus and Other Arenaviruses. Springer-Verlag, Berlin, Heidelberg and New York, 1973 p. 207 216.

13. *Marker O & Volkert M* Studies on cell-mediated immunity to lymphocytic choriomeningitis virus in mice. J Exp. Med. *137* 1511–1525 1973

14 *Marker O Andersen G T & Volkert, M.* The interplay between target organ concentrations of lymphocytic choriomeningitis virus and cell-mediated immunity in baby mice. Acta path. microbiol. scand. Sect. C, *84* 25–30 1976.

15 *Schmitt-Verhulst A. M & Shearer G M* Bifunctional major histocompatibility-linked genetic regulation of cell-mediated lympholysis to trinitrophenyl-modified autologous lymphocytes J Exp. Med. *142* 914–927 1975

16. *Shearer G M Rehn T G & Garbarino C. A.:* Cell-mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. Effector cell specificity to modified cell surface components controlled by the H 2K and H-2D serological regions of the murine major histocompatibility complex. J Exp. Med. *141* 1348–1363, 1975.

17 *Zinkernagel, R. M & Doherty P C.* Characteristics of the interaction in vitro between cytotoxic thymus-derived lymphocytes and target monolayers infected with LCM Scand. J Immunol. *3* 287 294 1974

18. *Zinkernagel, R M & Doherty P C* Re-

striction of in vitro T cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature *248* 701–702, 1974

19 *Zinkernagel R M & Doherty P C.*, Peritoneal macrophages as target cells for measuring virus-specific T cell mediated cytotoxicity in vitro J Imm. Meth. *8* 263–266 1975

20 *Zinkernagel, R. M & Doherty P C* H 2 compatibility requirement for T cell mediated lysis of target cells infected with lymphocytic choriomeningitis virus. J Exp Med. *141* 1427–1436 1975

21 *Zinkernagel, R M Dunlop M B C & Doherty P C* Cytotoxic T cell activity is strain-specific in outbred mice infected with lymphocytic choriomeningitis virus. J Immunol. *115* 1613–1616, 1975

Acta path. microbiol. scand. Sect. C, 84 455–464 1976

C1 SUBCOMPONENT COMPLEXES IN NORMAL AND PATHOLOGICAL SERA STUDIED BY CROSSED IMMUNOELECTROPHORESIS

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Laurell, A. B., Mårtensson, U & Sjöholm, A. G. C1 subcomponent complexes in normal and pathological sera studied by crossed immunoelectrophoresis. Acta path. microbiol. scand. Sect. C, 84 455–464 1976.

Selected pathological sera gave three molecular species of C1 protein on crossed immunoelectrophoresis in the presence of calcium. C1 precipitates were obtained at the origin and in the β_1 and regions. 12 normal sera gave C1 protein peaks at the origin and in α position. One of the normal sera also contained a small amount of the β C1 protein. The C1s protein at the origin represented macromolecular C1. The α peak was a complex composed of C1 IA, C1s and C1 proteins. This complex was preformed in serum and did not show C4 cleaving activity. The molecular species in the β region was shown to be calcium-dependent complex of C1r and C1s, probably in proenzyme form. The C1 -C1 complex formed macromolecular C1 on addition of purified C1q to serum. During electrophoresis activation of C1 subcomponents was initiated by mechanism involving C1 with generation of C1 activity in eluted fractions corresponding to the position of macromolecular C1 as well as in the β region. The significance of β C1 complexes or of α C1 complexes in normal and pathological sera was discussed.

Key words C1 subcomponent complexes crossed immunoelectrophoresis.

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C1 in normal human serum is a macromolecular complex of C1q, C1r and C1s, whose integrity is dependent on calcium ions (18). Interactions between purified C1q, C1r and C1s have been studied by crossed immunoelectrophoresis (15). Recently evidence was presented to suggest that the intact C1 complex contains C1t, a previously unrecognized plasma protein (1).

Aberrations of the C1 subcomponents have been reported in sera from patients with chronic urticaria and angioedema (16). In the present investigation it was shown that the C1r and C1s proteins in normal human serum are not exclusively associated with macromolecular C1 even in the presence of calcium. Analysis of pathological sera facilitated the characterization of C1r and C1s protein not bound to macromolecular C1. The enzymatic activity of fractions containing C1s protein was assessed by inactivation of hemolytic C4.

MATERIALS AND METHODS

Serum samples Sera from 17 apparently healthy individuals and from 5 patients with various diseases (Table 1) were frozen at −80 C within 4 hours of sampling The sera were stored in aliquots at −80 C until analyzed

Purification of complement components. C1q was prepared as described by *Yonemasu & Stroud* (35) and C1s according to *Haines & Lepow* (9) C1r protein was isolated from euglobulin obtained at pH 7 5 (32) by chromatography on DE 32 cellulose in accordance with the procedure devised by *Lepow et al.* (18) C1r was purified further by gel filtration on Sephadex G 200 in the presence of EDTA and by preparative agarose electrophoresis (11) The C1r preparation was hemolytically active (5) It contained about twice the normal serum concentration of C1r as determined by electroimmuno-assay C1 inactivator (C1 IA) was purified according to *Pensky et al.* (24)

Preliminary experiments indicated that the preparation of stable, functionally purified C2 was facilitated by carrying out the separations in the presence of calcium. Under such conditions it was also possible to obtain functionally pure C4 Pseudoglobulin was prepared according to *Wagner & Röllinghoff* (34) but in the presence of 0 001 M calcium. This concentration of calcium was used also in the chromatography buffers. C4 and C2 were completely separated by chromatography on CM 11 cellulose at pH 5 4 The C4 was rechromatographed under similar conditions. C2 was precipitated with Polyethylene glycol 6000 (PEG) (Kebo AB Stockholm, Sweden) at 8–15 per cent w/ The C2 was further purified by chromatography on DE 32 cellulose at pH 8.0 The concentrated C4 and C2 preparations contained about 30 per cent and 200 per cent, respectively of the normal serum concentrations as determined by electroimmuno-assay and by hemolytic titration (26)

Antisera. Rabbit antiserum against C1r was obtained by one subcutaneous dose of C1r emulsified in Freunds complete adjuvant After absorption the antiserum gave a single line in the Ouchterlony test against human EDTA plasma On immunoelectrophoresis and crossed immunoelectrophoresis a single precipitate in the beta region was obtained The specificity of the antiserum was examined by anti-C1r kindly placed at our disposal by Dr *R Stroud* University of Alabama, Birmingham, Alabama 35233 USA Anti-C1q anti-C1s anti-C1 IA anti-C3 and anti-C4 were prepared as described earlier (29)

Immunochemical determinations of complement components. C1q, C1s, C3 C4 and C1 IA (9) were quantitated by electroimmunoassay (17) as well as C1r and C2 (to be published)

Crossed immunoelectrophoresis. The procedure described by *Ganrot* (6) was used. Unless otherwise stated, the initial electrophoretic separation was performed with 0 075 M barbital buffer containing 0 002 M calcium lactate. In the second electrophoretic step barbital buffer containing 0 002 M EDTA was used, and the electrophoresis was carried out at low voltage (3–4 V/cm) for about 18 hours. In some experiments calcium-containing buffer was used in both the electrophoretic steps. When C1q was analyzed by crossed immunoelectrophoresis the second step was performed with 0 1 M phosphate buffer pH 6 0 containing 0 002 M EDTA.

Sephadex chromatography Gel filtration on Sephadex G 200 was carried out in 2.5 × 100 cm columns using 0 015 M triethanolamine buffer pH 7 4 with 0 135 M sodium chloride and 0.001 M calcium.

Enzyme inhibitors Heparin (5000 IU/ml Vitrum Stockholm, Sweden) *Sp 54* (100 mg/ml, Firma Bene-Chemie, Munich, Germany) and *Trasylol* (5 × 10⁶ KIE, Bayer AG Leverkusen, Germany) were added to serum before electrophoresis (30) *Soybean trypsin inhibitor* (SBTI) (Worthington Biochemical Sales Co., Freehold New Jersey USA) was used also in the gel medium. Some of the inhibitors were tried in functional hemolytic tests of C4 inactivation. *Carrageenan* (Sigma St. Louis, Missouri USA) treatment of serum was performed according to *Borsos et al.* (3)

C4 inactivation by electrophoretic fractions. EA C1 cells were prepared using IgM amboceptor and guinea pig C1 (32) and C4 titrations were made according to the technology given by *Rapp & Borsos* (26) After electrophoretic separation agarose strips 2 mm wide were cut out of the gel and eluted over night at 4 C in isotonic barbital buffer with calcium and magnesium. Aliquots of the eluates were incubated with C4 for one hour at 30 C followed by C4 titration in a total reaction volume of 1.3 ml In some experiments eluates were preincubated 15 minutes at 37 C with enzyme inhibitors, or elution was carried out in the presence of enzyme inhibitor At the final concentrations used the inhibitors did not interfere with the hemolytic assay system.

RESULTS

The electrophoretic distribution of C1r and C1s C1r and C1s proteins in 12 normal sera were analyzed separately by crossed immunoelectrophoresis Under the conditions used most of the C1r and C1s protein gave well defined narrow precipitates at the origin. In addition, a C1s protein peak in the α_2 region was invariably present (Fig 1) One of the

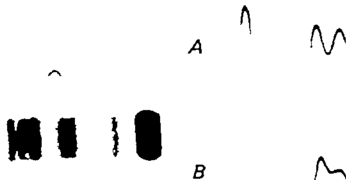


Fig 1 Crossed immunoelectrophoresis of normal human serum. Ca^{++} was present in the separating step. The second electrophoretic step was done in the presence of EDTA and with anti C1s in the gel. Anode to the right in the separating step and to the top in the second step.

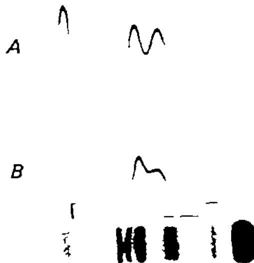


Fig 2 Crossed immunoelectrophoresis of two pathological sera. Ca^{++} was present in the separating step. The second electrophoretic step was done in the presence of EDTA and with anti C1 in the gel. A Serum B.H. B Serum M.W

normal sera gave C1r and C1s peaks in the same position in the fast β region. The β C1s precipitate identified with the α_2 C1s immunoprecipitate. The other sera showed cathodal extension of the α_2 precipitate into the β_1 region.

Five selected pathological sera (Table 1) with abnormal complement component levels were analyzed. Three molecular species of C1s protein were clearly distinguished giving precipitates at the origin in the β and in the α_2 regions (Fig. 2 A) C1r was found at the origin and in β position. Sera M.W B.M. and B.H. with dissociation of the C1r and C1s levels compared to C1q gave well defined C1r and C1s peaks in the β_1 region.

TABLE 1 *Pathological Sera Studied in the Investigation. Complement Component Levels Are Given in Percentages of the Concentrations in a Normal Standard Pool*

	C1q	C1	C1s	C1 IA	C4	C3	Clinical data
G K	50	67	85	25	3	98	Hereditary angioedema during attack.
J S	80	92	108	32	52	63	Hereditary angioedema trait. Asymptomatic child.
M W	< 1	102	131	144	25	51	Chronic urticaria. Asthma.
B M	48	127	124	180	365	190	Immunodeficiency syndrome. Hypogammaglobulinemia.
B H	90	158	182	151	59	70	Urticaria and angioedema during attack.
Normal	76 136	71 133	72 146	72–153	53–207	70–136	

MATERIALS AND METHODS

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A



B



C



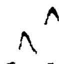


Fig. 4 Crossed immunoelectrophoresis of HANE serum J.S. (A and B) and of a mixture of purified C1 with purified C1 IA (C). Ca^{++} was present in the separating step. The second electrophoretic step was done in the presence of EDTA. A Anti C1 in the gel. B and C Mixture of anti C1 IA and anti C1 (the same concentration as in A)

thought that the α C1s peak might be a complex composed of C1s and C1 IA, normal sera and sera with high α C1s peaks were analyzed with the use of anti-C1s and different concentrations of anti-C1 IA in the gel. The patterns obtained suggested partial identity between the α_4 C1s protein and C1 IA, but the interpretation was invalidated by the presence of native C1 IA in the α_4 region. However it was clearly shown that the α_4 C1s peak in serum J.S. from an individual with the hereditary angioedema trait contained C1 IA (Fig. 4). In contrast to macromolecular C1 and to the C1r-C1s complex, the complex between C1 IA and C1s protein did not dissociate in the presence of EDTA.

Purified C1 IA incubated with C1s gave rise to a molecular species differing in mobility and appearance from the α_4 C1s complex on crossed immunoelectrophoresis (Fig. 4 C). The α_4 peak was therefore not thought to consist solely of C1s and C1 IA. However attempts to detect further components, in-

A





B

C





Fig. 5 Crossed immunoelectrophoresis of serum B.H. Both electrophoretic steps were run in the presence of Ca^{++}. A Anti C1s in the gel. B Anti C1. C Mixture of anti C1s and anti C1r in the same concentrations as in A and B.

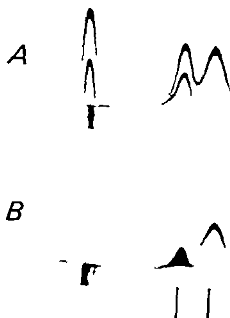


Fig 3 Crossed immunoelectrophoresis of the pathological serum B.H Ca^{++} was present in the separating step. The second electrophoretic step was done with a mixture of anti C1r and anti C1s in the gel and with A EDTA, B Ca^{++} in the buffer β_1 and α_2 zones are indicated by vertical lines.

The β_1 C1s identified with the α_2 C1s precipitate. Serum B.M with moderately reduced C1q levels showed low C1r and C1s peaks at the origin Serum M W with a C1q level <1 per cent showed no C1r or C1s at the origin (Fig 2 B) The two hereditary angio-edema sera G K and J.S gave no β_1 precipitate, but a pronounced α_2 peak in addition to the C1r and C1s at the origin

Gel filtration on Sephadex G 200 Serum M W with C1q <1 per cent was fractionated on Sephadex G 200 in the presence of calcium. As determined by electroimmunoassay C1s emerged in the first peak of the chromatogram. Analysis by crossed immunoelectrophoresis of the concentrated fractions containing C1s gave precipitates in the fast β and the α_2 regions. The β_1 peak was slightly anodal to the corresponding peak in serum

Separation on Sephadex G 200 of normal serum and normal carrageenin treated serum also gave C1s protein in the first peak of the chromatogram. Subsequent analysis by crossed immunoelectrophoresis showed that normal serum gave C1s at the origin and in the α_2 region. Carrageenin treated serum gave only the α_2 C1s protein peak.

Composition of the C1s containing complexes analysed Sera B.M and B.H. were analysed by crossed immunoelectrophoresis with a mixture of anti-C1r and anti-C1s, the second electrophoretic step being performed in the presence of EDTA. Distinct double peaks appeared at the origin and in the β_1 region. In the α_2 region a single precipitate was seen identifying with one of the β_1 peaks (Fig 3 A)

C1s protein at the origin. Analysis of C1q by crossed immunoelectrophoresis showed C1q at the origin in the same position as C1r and C1s provided the first electrophoretic step was performed in the presence of calcium and the second in the presence of EDTA. When both the electrophoretic steps were carried out in the presence of calcium no C1q C1r or C1s precipitates appeared (Fig 3 B) This indicated that C1q C1r and C1s in serum formed a calcium-dependent complex that did not migrate in the electric field under the conditions used In the presence of EDTA the C1qrs complex (i.e. macromolecular C1) dissociated, making possible the detection of the three subcomponents.

C1s protein in the β_1 region. When the second electrophoretic step was performed in the presence of calcium and with a mixture of anti-C1r and anti-C1s in the gel a single peak was seen in the β region showing partial identity with the α_2 peak. The peak stained inside its contour line indicating complex formation (17) (Fig 3 B) Increased amounts of anti-C1r or anti C1s in the gel reduced the height of the β_1 peak. This together with the finding of separate C1r and C1s β precipitates in the presence of EDTA proved that the peak represented a calcium-dependent complex between C1r and C1s protein.

C1s protein in the α_2 region As it was

lently activation of C1s occurred during elution of serum fractions by a mechanism that was inhibited by Liquoid.

DISCUSSION

In the present investigation it was shown by crossed immunoelectrophoresis that part of the C1r and C1s proteins in normal serum are not associated with the macromolecular C1 complex even in the presence of calcium. Thus, C1s protein appeared in α_2 position and together with C1r also in the β region. Some pathological sera had increased amounts of C1r and C1s not bound to C1q.

The finding of a calcium-dependent complex of C1q, C1r and C1s in serum at the origin is in accordance with that in earlier studies using purified preparations of these proteins (15). Analysis by crossed immunoelectrophoresis gave conclusive evidence that the C1r and C1s precipitates in the β region represent a calcium-dependent complex between these proteins in serum. On Sephadex G 200 the complex emerged in the first peak of the chromatogram. In contrast, *Valet & Cooper* (33) showed that purified proenzyme C1r and C1s formed a firm calcium-dependent 10 S complex in a molar ratio of 1 1. Work is in progress to characterize the C1r C1s complex in serum further.

After gel filtration the C1r-C1s complex showed a slightly increased electrophoretic mobility compared with that of the C1r-C1s complex in serum, and was capable of inactivating hemolytic C4. Activation of the C1r C1s complex apparently occurred during gel filtration. Evidence for the presence of a C1 activator in the macromolecular fraction has been reported by *Laurell* (14).

The α C1s protein peak in hereditary angioedema serum was clearly shown to be a complex between C1s protein and C1 IA. Owing to technical difficulties the presence of native C1 IA with α_2 mobility hampered interpretation when the α C1s in normal serum was analyzed.

The α_2 C1s peak in normal or carrageenin-treated normal sera appeared in the first peak on Sephadex G 200, well separated from native C1 IA. This material was shown to be a complex containing C1s protein and C1 IA. The α C1s complex, however was not identical with the complex formed between purified C1s and C1 IA, which suggested that it might not simply be a complex of these two proteins. In the presence of calcium in the second electrophoretic step the α_2 C1s peak was weakly stained or diffusely outlined, unless anti-C1s was combined with anti-C1r in the gel. Under these conditions anti-C1r also reduced the height of the α C1s peak. Although a C1r precipitate in the α_2 region was not seen when only anti-C1r was used, it was concluded that the α complex contained C1r protein in addition to C1s and C1 IA. Dissociation of the complex or disproportion between antibody and C1r determinants available in the complex might possibly explain the findings. Preliminary data in our laboratory indicating that purified C1r reacts poorly with C1 IA in the presence of calcium may be relevant in this context.

α_2 complexes were found in all the sera studied. A question is whether the α complex is preformed in serum or is generated during the electrophoresis. C1 IA reacts with C1r (27 36) and C1s (10 7). There is no evidence that C1 IA reacts with the proenzymes. The present finding that inhibitors of C1 activation and carrageenin-treatment of serum did not influence the appearance of the α_2 precipitation peak supports the view that the α complex was preformed in serum and not produced during electrophoresis.

Our data obtained with all sera studied indicated that the α_2 complex consisted of C1 IA bound to C1s and C1r probably also in the activated form. The C1r-C1s complex in the β region might not be the source of the α_2 complex since we found that the β_1 C1r C1s complex appeared in the non-active form in native serum. If the C1r-C1s complex were activated in serum the C1 IA should be bound to the complex, and no complexed C1r-C1s molecules should be found in the β_1 region on electrophoresis. The weight of the evidence thus suggests that the C1 subcompo-

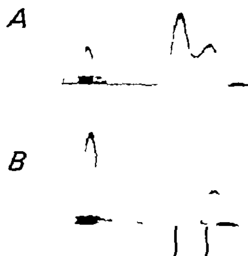


Fig 6 Crossed immunoelectrophoresis of serum B.M. A before and B after incubation with purified C1q. Ca^{++} was present in the separating step and EDTA in the second electrophoretic step

cluding C1r in the complex were unsuccessful when EDTA was present in the second electrophoretic step

On crossed immunoelectrophoresis of serum B M or B H in the presence of calcium in both electrophoretic steps with anti C1s in the gel the α_2 peak stained weakly and was in some of the experiments also diffuse. On analysis with a mixture of anti-C1r and anti C1s under these conditions the α_2 precipitate stained more intensely it was sharply outlined and clearly reduced in height (Fig 5) Although C1r protein in the α_2 region could not be detected by anti-C1r alone the findings suggested the presence of C1r protein in the α_2 C1s complex. Analysis of the α_2 C1s protein obtained by gel filtration on normal serum and of carrageenin treated normal serum likewise indicated that it was a complex made up of C1s, C1r and CI IA proteins.

Addition to serum of purified C1q Addition of purified C1q to sera with β C1r and C1s protein peaks resulted in the disappearance of these peaks and a concomitant increase of the C1r and C1s precipitates at the origin. No change of the α_2 C1s precipitate was seen (Fig 6)

Influence of enzyme inhibitors on C1s protein patterns Enzyme inhibitors were added to normal serum and to serum B.H. before electrophoresis. Heparin and Sp 54 in doses large enough to block C4 conversion (30) did not influence the appearance of the C1s precipitates in the β_1 and α_2 regions. Liquoid produced a diffuse β_1 C1s peak, but did not change the α_2 peak. The addition of SBTI to serum and to the gel medium (30) had no effect on the C1s protein pattern. Repeated carrageenin treatment of normal serum did not affect the α_2 C1s protein but resulted in disappearance of the C1s precipitate at the origin.

C4 inactivation by fractions from agarose electrophoresis Normal serum separated by agarose electrophoresis in the presence of calcium gave C4 inactivating fractions extending from the application site to the β-region. Maxima corresponding to the position of macromolecular C1 and to the β region were distinguished. The same pattern was obtained on analysis of sera B M and B.H. Serum M W with C1q <1 per cent gave no C4 inactivation. However after gel filtration of serum M W the isolated C1r C1s complex showed C4 cleaving activity Normal serum depleted of C1 by repeated carrageenin treatment did not give rise to electrophoretic C4 inactivating fractions.

The α_2 C1s complex isolated from native CI IA on Sephadex G 200 did not inactivate C4

Influence of enzyme inhibitors on C4 inactivating fractions Incubation with CI IA (about 10 per cent of serum concentration) of fractions eluted in buffer inhibited C4 inactivation. Liquoid (0 05 mg/ml) SBTI (10 mg/ml) and Trasylol (250 KIE/ml) were not effective. The results indicated that C4 inactivation was most likely mediated by C1s present in the eluted fractions.

On the other hand, when serum fractions were eluted in buffer containing Liquoid at 0 05 mg/ml the evolution of C4 cleaving activity was almost completely blocked Ev-

interaction between Carrageenin and the first component of complement. J Immunol. *94* 662–666, 1965

4. *Bosco M M E, De & Manni, J A.:* C1q, C1 and C1s titres in various clinical states. Medicina (Bs. Aires) 32 717 1972.

5. *Bruce M M E, De & Strand R M* C1r subunit of the first complement component purification, properties and assay based on its linking role. J clin. Invest. *50* 838–848, 1971

6. *Ganrot, P O* Crossed immunoelectrophoresis. Scand J clin. Lab. Invest. *29* suppl 124 39–47 1972.

7. *Gigli, I Ruddy S & Austen K. F* The stoichiometric measurement of the serum inhibitor of the first component of complement by the inhibition of immune hemolysis. J Immunol. *100* 1154–1164 1968.

8. *Hadjiyannaki, K & Lachmann P J* Hereditary angio-oedema review with special reference to pathogenesis and treatment. Clin. Allergy *1* 221 233 1971

9. *Haines, A. L. & Lepow I H* Studies on human C'1 esterase. I Purification and enzymatic properties. J. Immunol. 92 456–467 1964.

10. *Haines, A. L. & Lepow I H* Studies on human C'1 esterase II Function of purified C'1 esterase in the human complement system J. Immunol. *92* 468–478, 1964

11. *Johansson, B G* Agarose gel electrophoresis. Scand J clin Lab. Invest 29 suppl. 124 7–19 1972

12. *Kohler P F & Müller-Eberhard H J* Complement-immunoglobulin relation Deficiency of C'1q associated with impaired immunoglobulin G synthesis. Science *163* 474–475, 1969

13. *Kohler P F & Muller Eberhard H J* Metabolism of human C1q Studies in hypogammaglobulinemia, myeloma and systemic lupus erythematosus J clin Invest. 51 868–875 1972

14. *Laurell, A.-B.* An activator of C'1 to C'1 esterase in the macroglobulin fraction of human sera. Acta path microbiol. scand *77* 291 298, 1969

15. *Laurell, A -B. & Mårtensson U* Interaction between C1q, C1 and C1 from human serum. Acta path microbiol scand. Sect *B*, *82* 585–589 1974

16. *Lau B, A B Mårtensson, U & Sjöholm A G* Complement components in hereditary angioedema and chronic urticaria Int Archs Allergy appl Immunol. *49* 86–88, 1975

17. *Laurell, C B.* Electroimmuno assay Scand J clin Lab. Invest 29 Suppl 124 21 37 1972

18. *Lepow I H Naff G B., Todd, E. W Pensky J & Hinz, C F., J.* Chromatographic resolution of the first component of human complement into three activities. J exp. Med. *117* 983–1008, 1963.

19. *Loos, M., Borsos, T & Rapp H J* Activation of the first component of complement. Evidence for an internal activation step. J Immunol. *108* 683–688, 1972.

20. *Naff G B. & Ratnoff O D* The enzymatic nature of C1r Conversion of C1s to C1 esterase and digestion of amino acid esters by C1r J exp. Med *128* 571–593, 1968.

21. *Ablin J E & Ganrot P O* Plasmin, plasmin inhibitors and degradation products of fibrinogen in human serum during and after intravenous infusion of streptokinase Scand. J clin. Lab. Invest. *20* 113–121 1967

22. *Ohlsson K.* Interactions in vitro and in vivo between dog trypsin and dog plasma protease inhibitors. Scand. J clin Lab. Invest. *28* 219–225 1971

23. *Ohlsson K* Elimination of [125]I-trypsin-α-macroglobulin complexes from blood by reticuloendothelial cells in dog. Acta physiol. scand. *81* 269–272, 1971

24. *Pensky J Levy L R & Lepow I H.* Partial purification of serum inhibitor of C'1 esterase. J biol. Chem. *236* 1674–1679 1961

25. *Pickering, R J Naff G B., Stroud R M., Good R. A. & Gewurz, H* Deficiency of C1r in human serum Effects on the structure and function of macromolecular C1 J exp. Med. *131* 803–815, 1970.

26. *Rapp H J & Borsos T* Molecular basis of complement action Appleton-Century-Crofts, New York. 1970

27. *Ratnoff O D Pensky J Ogston D & Naff G B* The inhibition of plasmin, plasma kallikrein, plasma permeability factor and the C1 subcomponent of the first component of complement by serum C1 esterase inhibitor J exp Med *129* 315–331 1969

28. *Rosen, F S., Alper C A Pensky J Klemperer M R. & Donaldson, V H* Genetically determined heterogeneity of the C1 esterase inhibitor in patients with hereditary angioneurotic edema. J clin Invest. *50* 2143–2149 1971

29. *Sjöholm A G* Complement components in normal serum and plasma quantitated by electroimmunoassay Scand J Immunol. *4* 25–30 1975.

30. *Sjöholm A G & Laurell A. B.* Conversion of the fourth complement component studied by crossed immunoelectrophoresis. Clin. exp. Immunol. *14* 515–529 1973.

31. *Stroud R. M., Nagaki, R., Pickering, R. J Gewurz, H Good R. A & Cooper M D* Sub-units of the first complement component

nents of the α_2 complex were derived from macromolecular C1 reflecting an *in vivo* activation of part of the macromolecular C1

The literature contains only few studies concerning the normal presence of activated C1 in serum. *Loos et al* (19) produced evidence that part of C1 is activated in diluted normal human serum. It has been pointed out (8 28) that the levels of CI IA in HANE during remission are lower than expected. This might suggest a continous activation of C1 regulated by available CI IA a possibility that might also be valid under physiological conditions. The α_2 complexes found in all the sera studied by us might reflect such a course of events.

Complexes of α_2 macroglobulin with plasmin (21) or trypsin (22 23) are rapidly cleared from the circulation. In analogy the α_2 C1s complex may represent the form in which activated C1r and C1s are normally eliminated *in vivo*. The possibility might also be considered that the α_2 complex is a transport form for activated C1 subcomponents. Compared with what was seen in normal sera the α_2 precipitation peak in pathological sera was high which might suggest accelerated activation of C1 subcomponents in disease.

Analysis of electrophoretic fractions with respect to C4 inactivating capacity showed that the α_2 complex was enzymatically inactive. All normal sera, as well as pathological sera containing macromolecular C1 and C1r C1s complexes, gave C4 inactivation in fractions derived from the origin and also in the β region. It is noteworthy that the pathological serum M W deficient in C1q did not give rise to C4 inactivating fractions on elution which clearly suggested that the C1r C1s complex was in proenzyme form.

Evidence was presented to show that C4 inactivation by eluted electrophoretic fractions was mediated by C1s. The C1s activity was barely detectable when elution was carried out in the presence of Liquoid a potent inhibitor of C1r (20). Apparently activation of C1s occurred mainly during the elution procedure by a mechanism involving C1r. The present data together with earlier findings (30) suggest that activation of C1 is initiated during electrophoresis, ensuing in limited C1s activity. The elucidation of the sequence of events leading to activation of C1 subcomponents on agarose electrophoresis and during elution was not considered to fall within the scope of the present investigation.

An important question concerns the origin of the C1r-C1s complexes in serum. The present finding that C1r-C1s complexes react with purified C1q to form macromolecular C1 emphasizes that C1r-C1s complexes represent a surplus of these proteins to C1q in serum. *De Bracco & Manni* (4) reported dissociation between the serum levels of C1q C1r and C1s with extremely low C1q values in cases of SLE rheumatoid arthritis and viral haemorrhagic fever. Dissociation of C1q and C1s concentrations also occurs in immunodeficiency syndroms (12 31). *Stroud et al.* (31) discussed the possibility of independent synthesis of C1q and C1s and suggested that these proteins might be synthesized by different types of cells.

It was pointed out by *Pickering et al.* (25) that the synthesis of C1s and C1r might be closely linked. One interpretation is that increased *in vivo* elimination of C1q (13 2) creates a situation in which C1r and C1s are produced in excess of the C1q available for the assembly of macromolecular C1. The significance of C1r-C1s complexes in immunopathological conditions remains to be clarified.

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REFERENCES

1 *Assimeh S N & Painter R. H* The identification of a previously unrecognized subcomponent of the first component of complement J Immunol. 11? 482–487 1975

2 *Bokisch V A Top F H Jr Russell, P K. Dixon F J & Muller Eberhard H J* The potential pathogenic role of complement in dengue hemorrhagic shock syndrome. New Engl. J Med. 289 996–1000 1973

3 *Borsos T Rapp H J & Cruler C* The

Acta path. microbiol. scand. Sect. C, 84 465–470 1976

EFFECT OF SODIUM-SALICYLATE ON THE FUNCTION OF CULTURED, HUMAN MONONUCLEAR CELLS

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Viken, K. E. Effect of sodium-salicylate on the function of cultured, human mononuclear cells. Acta path. microbiol. scand. Sect. C, 84 465–470, 1976.

The in vitro effect of Na-salicylate on some functions of human mononuclear cells was studied. In therapeutical concentrations the drug was found to interfere both with the function of lymphocytes and monocytes/macrophages. Na-salicylate in concentrations of 400–800 μg/ml slightly inhibited the digestion of yeast particles. When the drug was present in the culture medium in doses above 160 μg/ml during the cell differentiation period from 90 minutes to the 8th day of culture, a reduction in the number of adhesive, viable cells was recorded. The remaining cells, however, were found to have a normal phagocytic function. A strong and dose dependent inhibition of the ability of lymphocytes to proliferate after antigenic stimulation with BCG bacilli was recorded. The inhibitory effect on the PHA response, however, was less prominent. The results presented indicate that Na-salicylate has a direct inhibitory effect on lymphocyte proliferation and monocyte differentiation and phagocytosis, which may be part of the explanation of the anti-inflammatory effect of the drug.

Key words: Na-salicylate; mononuclear phagocytes; lymphocytes; in vitro.

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The anti-inflammatory effect of salicylates is still not fully understood. Investigations have given some knowledge of isolated effects of the drug which may explain some aspects of its therapeutic effect. Salicylates together with other non-steroidal anti-inflammatory drugs have been shown to interfere with the carbohydrate metabolism of cells, mainly by uncoupling of oxidative phosphorylation (1). Inhibition of synthesis and release of endogenous substances such as vasoactive amines, which is of importance in the local inflammatory reaction, have also been suggested as one of the main therapeutic effects of salicylates (10). Salicylates have been found to stabilize isolated lysosome fractions, suggesting an inhibitory effect on phagocytic function and reduction of local injury caused by release of lysosomal enzymes from cells participating in the inflammatory process (4, 6). An inhibitory effect on the engulfment of latex particles (in vitro) in the presence of acetylsalicylic acid has been reported (5). The concentrations used in this experiment, however, were extremely high.

Recently an immunosuppressive action of salicylates has been suggested, based on the

in immunologic deficiency syndromes independence of C1s and C1q Clin exp Immunol *7* 133–137 1970

32. *Tamura N & Nelson R. A Jr* The purification and reactivity of the first component of complement from guinea pig human and canine sera. J Immunol. *101* 1333–1345 1968.

33 *Valet G & Cooper N R.* Isolation and characterization of the proenzyme form of the C1s subunit of the first complement component. J Immunol. *112* 339–350 1974

34 *Wagner H & Röllinghof M* C2—the second component of guinea pig complement purification and physicochemical characterization. Immunochemistry *7* 977–987 1970.

35 *Yonemasu K & Stroud R. M* C1q rapid purification method for preparation of monospecific antisera and for biochemical studies. J Immunol *106* 304–313 1971

36 *Ziccardi R J & Cooper N R.* Physicochemical and functional characterization of the C1r subunit of the first complement component. J Immunol. *116* 496–503 1976.

TABLE 1 *The Effect of Na-salicylate on Various Functions of Mononuclear Blood Cells During the First 90 Minutes of Culture*

Drug conc. μg/ml	Cellular adhesiveness	Survival	Engulfment capacity	Digestion capacity
800	90 ± 10	91 ± 4	92 ± 37	93 ± 20
400	100 ± 20	93 ± 6	98 ± 40	99 ± 12
160	93 ± 26	98 ± 10	102 ± 36	98 ± 16
80	100 ± 13	105 ± 14	103 ± 16	103 ± 2
40	99 ± 13	96 ± 6	108 ± 17	100 ± 4
0*	100*	100*	100*	100*

The figures listed are the means ± SD of the results from 6 experiments, carried out as described in test procedure 1 The results are expressed as per cent of control values. The experiments were performed in duplicate or triplicate petri-dishes.

* Control without drug addition Cellular adhesiveness 2 22 ± 0.53 range 1 40–2.78 × 10^4 adherent cells per culture dish Survival 550 ± 310 range 58–962 × 10^3 cells per culture dish after 8 days of culturing. Engulfment capacity 94 ± 36, range 40–138 × 10^3 ct/min, radioactivity per 2 coverslips. Digestion capacity 64 ± 4 per cent radioactivity found in the medium, range 58–67 per cent.

en calculated, and the results were expressed as percentages of control values without drug exposure. The figures presented are the mean values from 3–8 experiments ± standard deviation (SD) The p-values were calculated using the Wilcoxon Two-sample test

RESULTS

Na-salicylate was found to have no effect on the cellular adhesiveness of mononuclear cells in concentrations up to 800 μg/ml. A slight, but significant ($p<0.01$) decrease in the survival of macrophages 8 days after the short time exposure to Na-salicylate in concentrations of 400 and 800 μg/ml (Table 1) was recorded. The engulfment and digestion of radiolabelled *Candida albicans* were not found to be altered in cells treated in the same way (Table 1)

When testing macrophages cultured 8 days in the absence of the drug a slight, but significant ($p<0.05$) reduction of the engulfment capacity was recorded when the cells were exposed to Na-salicylate in concentration of 400–800 μg/ml during the engulfment period of 15 minutes. Exposure to Na-salicylate in concentrations exceeding 160 μg/ml resulted in a significant ($p<0.01$) inhibition of the intracellular digestion of the engulfed Candida particles (Table 2) Pre-incubation of the cells with Na-salicylate for half an hour and continuous exposure to the drug during the engulfment and digestive stages of phagocytosis, did not seem to increase this effect (Table 2)

The effect of Na-salicylate in various concentrations on the differentiation of monocytes to macrophages was also tested. A dose dependent reduction in the number of viable cells left on the 8th day of culture was recorded after exposure to drug concentrations exceeding 160 μg/ml. The total radioactivity per coverslip following addition of labelled Candida particles was found to be reduced proportionally to the reduction in the cell count, thus indicating no effect on the ability of the individual cell left to engulf the yeast particles (Table 3) The digestion capacity of the remaining cells was not found to be influenced by the long time exposure to Na-salicylate in the concentrations used (Table 3)

A strong and dose dependent inhibition of the incorporation of ^3H thymidine from the 5th to the 6th day of culture after BCG-stimulation was recorded. The effect was slightly more prominent when the cells were cultured in medium containing foetal bovine serum than with human AB serum (Table 4) The control data, however indicate that cul-

ability of salicylates to inhibit the prolifera-tion of lymphocytes after PHA-stimulation (7 9)

The aim of this investigation was to study the effect of sodium salicylate in therapeutic relevant doses on the function of human mononuclear phagocytes and lymphocytes using standardized *in vitro* procedures.

MATERIALS AND METHODS

Cell Separation

Human mononuclear cells were separated from defibrinated venous blood obtained from healthy volunteers as described earlier (15)

Monocyte Cell Culture

The mononuclear cells were suspended in medium R.P.M.I 1640 (Flow G.B.) supplemented with 25 per cent human, pooled A serum, 10 µg/ml glutamin and 50 µg gentamicin per ml. The cell concentration was adjusted to 3×10^6 cells per ml and the cell suspensions were dispensed in volumes of 2.5 ml per petri dish (diameter 5 cm, Nunclon, Denmark) or 0.5 ml per coverslip (11 × 35 mm) placed in duplicate in petri dishes.

Lymphocyte Cell Culture

The mononuclear cells were suspended in medium R.P.M.I 1640 supplemented with 20 per cent human pooled AB-serum or 20 per cent foetal bovine serum (Flow G.B.) and gentamicin and glutamin as described above. The cell concentration was adjusted to 1×10^6 cells per ml and dispensed in volumes of 2.5 ml per culture tube (Kimax)

Drug

Na salicylate (E. Merck A.G W G) was dissolved and diluted in medium R.P.M.I 1640 and sterilized by membrane filtration (Millipore 0 22 µm) before use. The general procedures for testing the cells have been described in detail in an earlier paper (14) The test procedures are summarized briefly

Test Procedure 1

The mononuclear cells were exposed to different concentrations of Na-salicylate during the first 90 minutes of culture. The cell layers were then washed once in Hanks balanced salt solution (BSS) before addition of fresh culture medium. The number of adhesive cells was estimated as the difference between the number of cells added and the number of cells removed after 90 minutes, counted in an electronic particle counter (Coulter type FN) The survival of the adhesive cells was recorded by counting the number of viable cells after 8 days of culture following this short time exposure to the drug, using an inverted phase contrast microscope (Reichert, Austria) The phagocytic ability of the remaining cells was tested by means of phagocytosis of heat killed, radiolabelled *Candida albicans* as described in detail earlier (11 12 14)

Test Procedure 2

Mononuclear phagocytes were cultured on cover slips for 8 days without drug addition before the test started. The engulfment and digestion stages of phagocytosis were measured separately after exposure to different concentrations of Na-salicylate as described in detail earlier (13 14) The phagocytic function was also tested after pre-incubation of the cells with Na-salicylate for half an hour before the test started The cells were also exposed to the drug continuously during the engulfment period of 15 minutes and the digestion period of 24 hours (13 14)

Test Procedure 3

The mononuclear cells were exposed to different concentrations of Na-salicylate during the cell differentiation period from 90 minutes to 8 days of culture. The drug was added at the standard medium changes at 90 minutes, 1 day and 4 days of culture. On the 8th day of culture, the number of cells left on the coverslips were counted in an inverted phase contrast microscope. The morphology of the cells was also studied. The phagocytic ability of the cells was then tested using radiolabelled *Candida albicans* as described earlier (13 14)

Test Procedure 4

The ability of lymphocytes to respond to antigenic and PHA (Gibco, USA) stimulation by proliferation during exposure to different concentrations of Na-salicylate was tested as described earlier (14) The antigen used was BCG-vaccine (Statens Seruminstitut Copenhagen) Cell proliferation was measured by means of ^{3}H thymidin-incorporation in a period of 18 hours from the 3rd to the 4th day of culture using PHA and from the 5th to the 6th day of culture using BCG (3) The cell cultures were harvested using a Titertek multiple cell harvester (Flow G.B.)

Statistics

The experiments were performed in duplicate r t pl at lture dishes or in fivefold culture tubes In each experiment the mean values from replicates containing the same drug concentration

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When testing macrophages cultured 8 days in the absence of the drug a slight, but significant ($p<0.05$) reduction of the engulfment capacity was recorded when the cells were exposed to Na-salicylate in concentration of 400–800 μg/ml during the engulfment period of 15 minutes. Exposure to Na-salicylate in concentrations exceeding 160 μg/ml resulted in a significant ($p<0.01$) inhibition of the intracellular digestion of the engulfed Candida particles (Table 2). Pre-incubation of the cells with Na-salicylate for half an hour and continuous exposure to the drug during the engulfment and digestive stages of phagocytosis, did not seem to increase this effect (Table 2).

The effect of Na-salicylate in various concentrations on the differentiation of monocytes to macrophages was also tested. A dose dependent reduction in the number of viable cells left on the 8th day of culture was recorded after exposure to drug concentrations exceeding 160 μg/ml. The total radioactivity per coverslip following addition of labelled Candida particles was found to be reduced proportionally to the reduction in the cell count, thus indicating no effect on the ability of the individual cell left to engulf the yeast particles (Table 3). The digestion capacity of the remaining cells was not found to be influenced by the long time exposure to Na-salicylate in the concentrations used (Table 3).

A strong and dose dependent inhibition of the incorporation of ^{3}H thymidine from the 5th to the 6th day of culture after BCG-stimulation was recorded. The effect was slightly more prominent when the cells were cultured in medium containing foetal bovine serum than with human AB serum (Table 4). The control data, however, indicate that cul-

TABLE 2. *The Effect of Na-salicylate on the Phagocytic Function of Macrophages Cultured 8 Days in Vitro in Absence of Drug*

Drug conc. µg/ml	Engulfment capacity		Digestion capacity	
	A	B	A	B
800	89 ± 18	84 ± 22	82 ± 5	83 ± 5
400	95 ± 18	87 ± 8	89 ± 4	91 ± 5
160	101 ± 24	92 ± 7	96 ± 4	95 ± 4
80	113 ± 18	98 ± 16	100 ± 4	97 ± 4
40	96 ± 10	100 ± 8	99 ± 6	99 ± 5
0	100*	100*	100*	100*

A Pre-incubated for half an hour with drug before test start.
B No pre incubation.
The figures listed are means ± SD of the results from A 8 – B 5 + 6 experiments, carried out as described in test procedure 2. The results are expressed as per cent of control values. The experiments were performed in duplicate and triplicate petri-dishes.
* Control without drug addition Engulfment capacity A 98 ± 16 range 78–121 × 10³ ct/min, radioactivity per culture dish, B 64 ± 20 range 47–94 × 10³ ct/min, radioactivity per coverslip. Digestion capacity A 70 ± 5 range 66–80 per cent B 65 ± 5 range 57–70 per cent radioactivity found in the medium.

ture medium containing FB-serum has a stimulating effect on lymphocyte proliferation at the magnitude of BCG itself When culture medium containing AB-serum was used BCG-induced lymphocyte proliferation was found to be inhibited by Na-salicylate.

TABLE 3 *The Effect of Na salicylate on the Differentiation of Monocytes to Macrophages*

Drug conc. µg/ml	Number of cells left after 8 days of culturing	Engulfment capacity	Digestion capacity
800	60 ± 17	61 ± 10	100 ± 4
400	72 ± 15	85 ± 16	102 ± 5
160	96 ± 6	102 ± 10	101 ± 4
80	102 ± 7	99 ± 11	101 ± 3
40	103 ± 9	98 ± 10	101 ± 4
0*	100*	100*	100

The figures listed are the means ± SD of the results from 6 experiments, carried out a described in test procedure 3 The results are expressed as per cent of control values. The experiments were performed in triplicate petri-dishes.
* Control without drug addition Number of cells left after 8 days 355 ± 95 range 281–545 × 10³ cells per culture dish. Engulfment capacity 116 ± 48, range 87–212 × 10³ ct/min radioactivity per culture dish. Digestion capacity 73 ± 6 range 64–81 per cent radioactivity found in the medium.

The effect of Na salicylate on PHA-stimulated proliferation of lymphocytes was found to be a slight inhibition of ³H thymidine incorporation in doses of 800 µg/ml when the cells were cultured in medium containing FB-serum ($p<0.01$) and AB-serum ($p<0.01$) The control figures show that FB-serum has a greater stimulatory effect than AB-serum, measured between the 3rd and 4th day of culturing (Table 4)

DISCUSSION

Mononuclear phagocytes and lymphocytes are important cells in chronic inflammation. It is therefore of interest to study the effect of a well known anti inflammatory drug as salicylate on some functions of these cells. As shown in this study Na salicylate in therepeutic concentrations did not influence the adhesiveness of monocytes to a plastic surface. Exposed to Na salicylate in concentrations above 160 µg/ml during the engulfment or digestion stage of phagocytosis the macrophages showed a small but significant decrease in the phagocytic function The presence of the drug

TABLE 4 The Effect of Na-salicylate on the Incorporation of ^{3}H-thymidine in Lymphocytes after Stimulation with BCG or PHA

Drug conc. µg/ml	% of control values			
	BCG/FB	BCG/AB	PHA/FB	PHA/AB
800	2 ± 1	6 ± 3	43 ± 14	80 ± 22
400	8 ± 5	30 ± 12	96 ± 16	83 ± 27
160	26 ± 16	56 ± 14	97 ± 12	99 ± 22
80	56 ± 23	71 ± 14	90 ± 18	99 ± 12
40	73 ± 23	77 ± 7	107 ± 29	98 ± 18
0	100	100	100	100
C*	146 ± 45	3 ± 2	26 ± 15	2 ± 1
	ct/min per culture tube × 10³			
0	163 ± 90	110 ± 62	183 ± 50	247 ± 84
Range	112–224	72–229	115–230	80–300

C* = control without addition of BCG or PHA.
The figures listed are the means ± SD of the results from 6 experiments in each group, carried out as described in test procedure 4. Each experiment was performed in 5-plicate culture tubes. FB: Culture medium containing 20 per cent foetal bovine serum. AB: Culture medium containing 20 per cent pooled AB-serum.

during the differentiation of monocytes to macrophages led to a reduced survival or at least a reduction in the number of adherent macrophages recorded on the 8th day of culturing. After removal of the drug, a normal phagocytic ability of the remaining cells was registered. According to this observation, Na-salicylate in therapeutic to toxic concentrations has a small depressive effect on the phagocytic function of the macrophages in vitro only when the drug is present in the medium during the test. The inhibitory effect seems to be reversed when the drug is removed.

The suppressive effect of salicylates on some functions of the cultured cells may in part be explained by interference of the drug with carbohydrate metabolism (9) and ATP production in the cells (1, 2). Inhibition of hexokinase activity as demonstrated in lymphocyte cultures (9) may lead to reduced glycolysis and reduced ability to produce energy from glucose. Uncoupling of oxidative phosphorylation (1, 2) may lead to reduced production of ATP from the respiratory chain. Both these effects may explain the reduction in energy-demanding functions, as the engulfment of particles and the ability of the cells to stay adherent to the coverslips during the differentiation of monocytes to macrophages. However, it is surprising that the same shortage of energy does not seem to interfere with the initial adherence of monocytes and the more energy demanding differentiation of monocytes to macrophages, involving for example the development of the lysosomal apparatus.

Pre-incubation of macrophages with Na salicylate was not found to increase the depressive effect on the digestive ability of the cells, compared to the effect of the drug on phagocytes in which fusion of lysosomes and phagosomes had occurred. Accordingly, lysosomal stabilization induced by salicylate could not be detected in our system, using lack of fusion as a measure for the term "stabilization". It seems more likely that salicylate has a direct inhibitory effect on lysosomal enzymes.

As regards the inhibitory effect of salicylates on the proliferation of lymphocytes after antigenic stimulation, Schneider et al. (9)

demonstrated an inhibition of the hexokinase activity as a possible explanation of this effect They also found that the inhibitory effect of acetyl salicylic acid could be reversed by increasing the concentration of glucose in the culture medium Uncoupling of oxidative phosphorylation leading to reduced production of chemically bound energy in the mitochondria may also contribute to the inhibition of the energy-demanding proliferation of lymphocytes. Inhibition of PHA response was not prominent in our study compared to the results of *Schneider et al* (9) and *Opelz et al.* (7) using acetylsalicylic acid. The inhibitory effect on the BCG response, however was identical to the inhibition of the PHA response in these studies.

The results in the lymphocyte stimulation experiments show an effect of methodological importance namely the stimulatory effect of foetal bovine serum on lymphocytes. A similar effect has also been reported in other test systems (8)

Technical assistance from Mrs. *B H Hansen* is gratefully acknowledged. I also want to thank professor *J O Lamvik* for assistance during the work and the preparation of the manuscript. The author was a research fellow of the *Norwegian Research Council for Science and the Humanities.* The work was supported by grants from the *Norwegian Research Council for Science and the Humanities* the *Norwegian Cancer Society* and the *Norwegian Society for Fighting Cancer*

REFERENCES

1 *Adams S S & Cobb R* A possible basis for the anti-inflammatory activity of salicylates and other non-hormonal anti rheumatic drugs Nature (Lond.) *181* 773–774 1958

2 *Brody T M* Action of sodium salicylate and related compounds on tissue metabolism in vitro J Pharm Exp Ther *117* 39 51 1956

3 *Hages D & Caspari E A* Lymphocyte transformation in vitro measured by tritiated thymidin uptake. I Lymphocyte culture techniques. Int. Arch. Allergy *37* 506–531 1970.

4 *Ignarro L J* Lysosome membrane stabilization *in vivo* Effects of steroidal and nonsteroidal anti-inflammatory drugs on the integrity of rat liver lysosomes. J Pharm. Exp. Ther *182* 179–188 1972.

5 *Kvarstein B & Stormorken H* Influence of acetylsalicylic acid, butazolidine, colchicine, hydrocortisone, chlorpromazine and imipramine on the phagocytosis of polystyrene latex particles by human leucocytes. Biochem. Pharmacol. *20* 119–124 1971

6. *Miller W S & Smith J G Jr* Effect of acetylsalicylic acid on lysosomes. Proc. Soc. Exp Biol *122* 634–636 1966.

7 *Opelz G Terasaki P I & Hirata A A* Suppression of lymphocyte transformation by aspirin. Lancet *i* 478–480 1973

8 *Rotkvam A & Heiby E. E.* Phagocytosis and microbicidal capacity of mouse macrophages non-specifically activated *in vitro* Acta path. microbiol. scand. Sect. C *83* 121–128, 1975

9 *Schneider W Papper A & Schernex P G* The effect of acetylsalicylic acid on the metabolism and transformability of human lymphocytes *in vitro* Klin Wochenschr *49* 1187–1189 1971

10 *Vane J R..* Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs Nature New Biology *231* 232–235 1971

11 *Viken K E* ^{125}I-labelling of *Candida albicans* by electrolysis. Acta path. microbiol. scand. Sect. B, *82* 219–222, 1974

12. *Viken K E. & Ødegaard A* Phagocytosis of heat killed radiolabelled *Candida albicans* by human blood monocytes cultured *in vitro* Acta path microbiol scand Sect. B *82* 235–244 1974

13 *Viken K E.* The effect of steroids on differentiation and function of cultured, mononuclear cells. Acta path. microbiol. scand. Sect. C, *84* 13–22 1976

14 *Viken K E & Lamvik J O* Effect of aurothiomalate on human mononuclear blood cells cultured *in vitro* Acta path. microbiol. scand. Sect C *84* 419–424 1976.

15 *Ødegaard A Viken K E & Lamvik J O* Structural and functional properties of blood monocytes cultured *in vitro* Acta path. microbiol scand Sect. C *82* 223–234 1974

SOME PHYSICOCHEMICAL PROPERTIES OF HUMAN LEUCOCYTE MIGRATION INHIBITORY FACTOR (LIF)

Evidence for the Participation of a Reactive Serine in the LIF Molecule

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Bendtzen, K. Some physicochemical properties of human leucocyte migration inhibitory factor (LIF). Evidence for the participation of a reactive serine in the LIF molecule. Acta path. microbiol. scand. Sect. C, 84: 471–476, 1976.

Leucocyte migration inhibitory factor (LIF) obtained from human lymphocytes stimulated with concanavalin A was consistently and irreversibly blocked by the serine-esterase inhibitor phenylmethyl sulphonylfluoride (PMSF). This effect was not due to fluoride ions, hydrolysis products of PMSF or to impurities. PMSF pulse treatment of human buffy coat cells did not affect cell migration under agarose. LIF was also irreversibly destroyed by treatment with L-cysteine and 2-mercapto-ethanol, suggesting that the molecule contains disulphide linkage groups decisive for its configuration and biological activity. Di-sodium EDTA completely inhibited LIF activity but only if present during the entire migration period. Removal of EDTA before LIF assay restored LIF activity. Leucocyte migration was neither influenced by L-cysteine nor by EDTA. LIF activity was slightly diminished after treatment at 56° C for 1 h and completely lost at 80° C for ½ h. Furthermore, LIF appeared rather stable when treated at pH values between 4 and 11. These findings suggest, but do not prove, an esterase or a protease nature of human LIF.

Key words: Leucocyte migration inhibitory factor (LIF); phenylmethyl sulphonylfluoride; cation requirement; disulphide bonds.

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Activated lymphocytes elaborate a number of chemical substances, lymphokines, which are thought to play a role in cell-mediated immunity. The biochemical characterization of lymphokines is still in its preliminary stages. Very little is known about the nature and the mode of action of these substances. Even macrophage migration inhibitory factor (MIF), one of the best studied lymphokines, has been characterized only in terms of some general physicochemical properties (14).

The unexpectedly low stability of human leucocyte migration inhibitory factor (LIF) (7) and the fact that even very small amounts of protein exhibit strong biological effects (5) led to the assumption that LIF might be a molecule with enzyme activity. The finding

of esterase activity in the supernatants of antigen stimulated lymphocytes (10) further motivated investigations of a possible esterase character of human LIF

This paper presents the results of initial studies on the effect of phenylmethyl sulphonylfluoride (PMSF) on LIF This agent irreversibly blocks LIF activity, suggesting the participation of a reactive serine in the LIF molecule. Furthermore findings implying that LIF contains disulphide bonds sensitive to L-cysteine and 2 mercaptoethanol, and that divalent cations are needed for the expression of LIF activity are reported

MATERIALS AND METHODS

LIF Production

LIF production and subsequent handling of lymphocyte supernatants was carried out as previously described (6) In brief human peripheral blood lymphocytes were incubated in the presence (LIF rich supernatant) or in the absence (control supernatant) of concanavalin A (Con A) 80 μg/ml (Pharmacia, Uppsala, Sweden) After culture the supernatants were harvested and the control supernatants were reconstituted with Con A. Supernatants were then desalted on small Sephadex G-50 columns (Pharmacia) lyophilized, pooled in LIF rich and control supernatants and chromatographed on Sephadex G-100 glass columns (1 6 × 90) in volatile buffer (0 05 M ammonium bicarbonate acetic acid pH 7 2) Fractions containing molecules of molecular weight between 40 000 and 80,000 daltons were pooled lyophilized and stored at 4 C until use.

Assay for LIF

The indirect leucocyte migration agarose technique originally described by *Clausen* (8) was employed using peripheral blood leucocytes as migratory cells. 22 × 10⁶ cells/90 μl culture supernatant were tested in 7 μl aliquots for migration under agarose and a migration index (MI) was determined

$$MI = \frac{\text{Mean area of migration in the presence of LIF-rich supernatant}}{\text{Mean area of migration in the presence of control supernatant}}$$

Mean area was determined by quadruplicate tests

Treatment of LIF with Inhibitor

Two samples of PMSF were used (Sigma, St. Louis, Mo., U.S.A. and Merck, Darmstadt, West Germany) A stem solution was prepared by dissolving PMSF in 2 propanole followed by dilution 1 9 with distilled water and a further dilution 1 9 with phosphate buffered saline, pH 7 2 (PBS) The final stem solution (1 per cent V/V 2-propanole and 10 mM PMSF) was either used immediately or kept at 4 C until use. In some experiments, PMSF was inactivated at pH 13.5 for 22 h at 37 C. The solution was brought back to pH 7 2 with 5 N HCl before use.

Lyophilized LIF rich and control supernatants each dissolved in 990 μl TC-199 (Hepes-buffered with antibiotics) were incubated with 10 μl PMSF stem solution for 30 min at 37 C (0 1 mM final concentration of PMSF) To test for initial lymphokine activity 990 μl LIF and control supernatants were added 10 μl PBS 1 per cent V/V 2 propanole and treated in parallel. After preincubation the PMSF was removed by dialysis at 4 C against distilled water (3 h) and Hanks balanced salt solution (HBSS) (40 h) before testing for LIF When tested on leucocytes, the final LIF concentration corresponded to two times that of the original supernatant.

The direct PMSF effect on leucocyte migration was tested in two ways A. TC-199 with and without 0.1 mM PMSF were pre-incubated and dialysed as described above. Subsequently leucocytes incubated in both solutions were allowed to migrate under agarose. B. Two aliquots of leucocyte suspensions were incubated at 37 C for 30 min in the presence or absence of 0 1 mM PMSF After washing three times with HBSS the cells were allowed to migrate. The migration index was calculated using the formula

$$MI = \frac{\text{Mean area of cell migration after PMSF treatment}}{\text{Mean area of cell migration in control}}$$

Treatment of LIF with Reducing Agents and Di Sodium Edetate (EDTA)

LIF rich and control supernatants were pulse treated for 30 min at 37 C with 10 mM 2-mercaptoethanol (Sigma) 10 mM L-cysteine, glutathione (reduced) and L methionine (Merck) and with 1 mM di sodium EDTA (Dispensary Rigshospitalet, Copenhagen Denmark) All agents were dissolved in PBS and adjusted to pH 7 2 before use. After pre-incubation the supernatants were dialysed and handled as described above prior to testing for LIF In some experiments, LIF activities were determined also in non-dialysed supernatants containing L-cysteine or EDTA.

Temperature and pH Stability of LIF

Lyophilized LIF-rich and control materials were dissolved in 0.05 M glycine buffers adjusted at different pH values (pH 3, 4, 10 and 11) with 1 N HCl or 1 N NaOH and incubated at 20° C for 30 min. The supernatants were then dialyzed against PBS for 4 h and against HBSS for 18 h at 4 C before LIF assay. As a control for the original LIF activity lyophilized supernatants were dissolved in PBS and examined in parallel.

The temperature stability of LIF was determined by heating the supernatants on water baths at 56° C and at 80° C for 30 min. Controls were examined in parallel at 37 C.

The inhibitory effect on LIF was calculated by comparing the MI of supernatants treated at extreme pH or temperature ($MI_{Treated}$) with the MI of untreated supernatants ($MI_{Control}$) using the formula.

$$\text{Per cent inhibition of LIF} = \frac{MI_{Treated} - MI_{Control}}{1 - MI_{Control}} \times 100.$$

RESULTS

Organophosphorus inhibitors such as di-isopropyl phosphorofluoridate (DFP) and various phosphonate esters as well as PMSF are all toxic to man due to the irreversible inhibition of esterases. It was therefore important to test the direct effect of PMSF on leucocyte migration. Preliminary experiments with 0.1 mM and 1 mM of PMSF added the leucocytes throughout the entire migration period showed only a borderline migration inhibition. However PMSF pulse treatment of supernatants as well as pulse treatment of indicator cells did not show any inhibition (Table 1) and cell viability as determined by trypan blue exclusion was not influenced either

PMSF pulse treatment of LIF-rich supernatants consistently abolished lymphokine activity (Table 2) The effect was irreversible since PMSF was removed prior to assay for LIF activity To clarify whether the action of PMSF might be mediated by hydrolysis products of PMSF or by fluoride ions, the inhibitor was subjected to alkaline hydrolysation and then assayed for LIF inhibitory effect. As shown in Table 2, this preparation did not affect LIF Furthermore, 0.1 mM sodium fluoride was inactive in blocking LIF

As to the molecular nature of LIF it was also interesting to determine the role of di-

TABLE 1 *The Effect on Cell Migration of PMSF Pulse Treatment of Supernatants and Indicator Cells*

		Agent	No. of exp	MI ± SD*	P
Supernatant	†	0.1 mM PMSF	5	0.97 ± 0.12	n.s.§
		0.1 mM PMSF (inactivated)	3	0.93 ± 0.06	–
Indicator cells		0.1 mM PMSF	5	0.96 ± 0.06	n.s.

* Migration index ± standard deviation.
§ P > 0.05 was considered not significant (Mann-Whitney rank sum test)

TABLE 2. *The Effect on LIF of PMSF Pulse Treatment*

Agent	No. of exp.	MI ± SD Control	MI ± SD Treated	P§
0.1 mM PMSF	6	0.69 ± 0.06	0.93 ± 0.07	<0.01
0.1 mM PMSF (inactivated)	3	0.72 ± 0.01	0.72 ± 0.04	–
0.1 mM NaF	2	0.73 ± 0.02	0.74 ± 0.06	–

Migration index ± standard deviation.
§ P values determined by Mann-Whitney rank sum test.

TABLE 3 *The Effect of L-cysteine and 2 mercapto-ethanol on Human LIF*

Agent	Pulse treatment	No of exp	MI ± SD* Control	MI ± SD Treated	P
10 mM L-cysteine	+	4	0 70 ± 0 08	0 94 ± 0 06	<0 05
	—	5	0 73 ± 0 08	0.91 ± 0.05	<0.02
10 mM 2 mercapto-ethanol	+	4	0 78 ± 0 06	0.89 ± 0 05	<0.05
10 mM Glutathione (reduced)	+	4	0 76 ± 0 05	0 78 ± 0 05	n.s.§
10 mM L methionine	+	4	0 76 ± 0 05	0 79 ± 0 04	n.s.

* Migration index ± standard deviation.
§ P > 0 05 was considered not significant (Mann Whitney rank sum test)

TABLE 4 *The Effect of Di-sodium EDTA on LIF*

Agent	Pulse treatment	No of exp.	MI ± SD* Control	MI ± SD Treated	P
1 mM di-sodium EDTA	+	4	0.69 ± 0 06	0 73 ± 0.02	n.s.§
	—	5	0 71 ± 0 09	0.98 ± 0 05	<0 01

* Migration index ± standard deviation.
§ P > 0 05 was considered not significant (Mann-Whitney rank sum test)

sulphide bonds in the expression of LIF activity The necessity for divalent cations for the activity of LIF was also examined.

As shown in Table 3 the reducing agents L-cysteine and 2 mercaptoethanole but not glutathione or the non reducing sulphur containing amino acid methionine, blocked LIF suggesting that the molecule contains disulphide linkage groups responsible for its configuration and biological activity Even pulse treatment at 37 C for 30 min was effective showing the irreversible nature of the blockade.

When LIF rich supernatants were tested in the presence of EDTA the mediator activity was also abolished. This effect, however was reversible since removal of EDTA by dialysis restored LIF activity (Table 4) No significant effect on cell migration was seen after treatment of leucocytes with either of these chemicals.

Finally the stability of LIF at extreme temperatures and pH was investigated. In three experiments LIF was partially inactivated at 56° C for 1 h and destroyed at 80° C for ½ h (Fig 1) In four different experiments, LIF proved rather stable when dissolved and treated for 30 min at pH values between 4 and 10 Even at pH 3 and at pH 11 LIF activity was only partially destroyed (Fig 1)

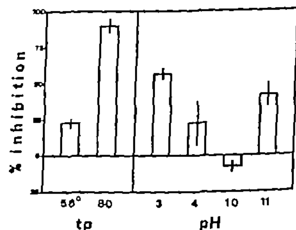


Fig 1 Per cent inhibition of LIF activity at extreme temperature and pH. Vertical lines indicate ± SD

DISCUSSION

The regulating functions of esterases in certain immune reactions have been known for some time. Thus, macrophage- and polymorphonuclear leucocyte associated esterases seem to determine the cell response to both guinea pig MIF and rabbit chemotactic factor (3, 13) the antigen induced release of histamine and slow-reacting substance of anaphylaxis from human lung, the antigen dependent, IgE-mediated release of histamine from human peripheral blood leucocytes and the immunological release of histamine from platelets (all reviewed in 3) have been shown to involve one or another esterase. Furthermore, evidence for a possible role of proteases in the mechanism of MIF action has been published recently (10) In these experiments, migration inhibitory activities present in both specifically and nonspecifically stimulated human lymphocyte supernatants could be blocked by various antiproteases of animal and plant origin and by the serine-esterase inhibitor DFP Lymphokines acting on human granulocytes as well as on monocytes were inhibited, suggesting that both LIF and MIF were blocked. Conflicting results, however have been obtained in thorough studies of guinea pig MIF which was not inhibited by DFP or by other organophosphorus esterase inhibitors (9)

The results presented here show that human LIF tested on unseparated human peripheral blood leucocytes was completely blocked by PMSF The effect was irreversible since removal of the inhibitor by extensive dialysis did not restore LIF activity Furthermore, when subjected to alkaline hydrolysis, the PMSF solution lost its inhibitory activity on LIF PMSF acts by changing a serine hydroxyl group to a phenylmethylsulphonyl group thereby blocking protein activities dependant on the serine hydroxyl group (12) This is particularly true of a number of ester hydrolyzing enzymes such as trypsin, chymotrypsin, thrombin etc Indeed, the results presented here and preliminary experiments performed in our laboratory indicate that human LIF acts as a protease of trypsin-like character (*Bendtzen* in preparation)

The activities of many proteins and enzymes depend on intact disulphide bonds in the molecule. Cysteine is capable of cleaving disulphide groups and guinea pig MIF can be blocked by treatment with cysteine, but not by cysteic acid (4) The total and irreversible blocking of LIF activity by cysteine and 2 mercaptoethanol suggests that disulphide bonds are present in the molecule and that these bonds are essential for the LIF activity However since reduced glutathione was not capable of blocking the mediator other mechanisms of LIF inactivation cannot be ruled out.

EDTA completely abolished LIF activity but had no effect on leucocytes migrating in medium alone. This effect was reversible, suggesting that LIF has an absolute metal cation requirement. Similarly aryl esterases are known to be inhibited by EDTA and activated by calcium ions (2) the antigen activated esterases associated with IgE-mediated release of histamine from leucocytes also require calcium ions for biological activity (3) It should be remembered, however that metal cations are involved in several cellular processes. Thus, human lymphocyte transformation (1) and guinea pig MIF (11) are also dependent on metal cations. Furthermore, divalent cations are also necessary for the activity of some non-enzyme molecules.

The present results suggest that human LIF exerts its biological effect through a reactive serine group and that the molecule may exhibit enzymatic activity The nature and location of the specific substrate for such an enzyme is obscure. One possibility would be a serum constituent split by LIF the split products in turn inhibiting migration. Recent experiments indicating the presence of specific receptors for human LIF on granulocytes (6) make this hypothesis less probable. A direct effect on target cells as a mechanism of action therefore seems more likely Experiments to elucidate the mechanism of action of LIF are now in progress.

TABLE 3 *The Effect of L-cysteine and 2 mercapto-ethanol on Human LIF*

Agent	Pulse treatment	No of exp	MI ± SD* Control	MI ± SD Treated	P
10 mM L-cysteine	+	4	0 70 ± 0 08	0.94 ± 0.06	<0.05
	—	5	0 73 ± 0 08	0.91 ± 0 05	<0 02
10 mM 2-mercapto-ethanol	+	4	0.78 ± 0.06	0.89 ± 0 05	<0 05
10 mM Glutathione (reduced)	+	4	0 76 ± 0 05	0 78 ± 0 05	n.s.§
10 mM L-methionine	+	4	0 76 ± 0.05	0.79 ± 0 04	n.s.

* Migration index ± standard deviation.
§ P > 0 05 was considered not significant (Mann Whitney rank sum test)

TABLE 4 *The Effect of Di-sodium EDTA on LIF*

Agent	Pulse treatment	No. of exp.	MI ± SD* Control	MI ± SD Treated	P
1 mM di-sodium EDTA	+	4	0 69 ± 0 06	0.75 ± 0.02	n.s.§
	—	5	0 71 ± 0 09	0.98 ± 0 05	<0.01

* Migration index ± standard deviation.
§ P > 0 05 was considered not significant (Mann Whitney rank sum test)

sulphide bonds in the expression of LIF activity The necessity for divalent cations for the activity of LIF was also examined

As shown in Table 3 the reducing agents L-cysteine and 2 mercaptoethanole but not glutathione or the non reducing sulphur containing amino acid methionine, blocked LIF suggesting that the molecule contains disulphide linkage groups responsible for its configuration and biological activity Even pulse treatment at 37 C for 30 min was effective, showing the irreversible nature of the blockade.

When LIF rich supernatants were tested in the presence of EDTA, the mediator activity was also abolished. This effect however was reversible since removal of EDTA by dialysis restored LIF activity (Table 4) No significant effect on cell migration was seen after treatment of leucocytes with either of these chemicals.

Finally the stability of LIF at extreme temperatures and pH was investigated In three experiments LIF was partially inactivated at 56° C for 1 h and destroyed at 80° C for ½ h (Fig 1) In four different experiments, LIF proved rather stable when dissolved and treated for 30 min at pH values between 4 and 10 Even at pH 3 and at pH 11 LIF activity was only partially destroyed (Fig 1)

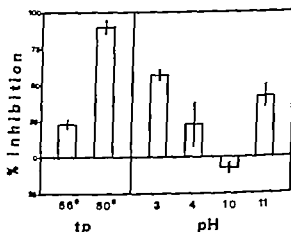


Fig 1 Per cent inhibition of LIF activity at extreme temperature and pH Vertical lines indicate ± SD

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LYMPHOCYTE MARKERS IN NEWBORN INFANTS

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Hallberg, T. & Hallberg, A. Lymphocyte markers in newborn infants. Acta path. microbiol. scand. Sect. C, 84: 477–484, 1976.

Purified peripheral blood lymphocytes from 20 newborn infants and 12 adults have been studied for the presence of surface markers. Adults and infants did not differ in the percentage of sheep RBC-binding cells (means 58–65 per cent) or in Ig-bearing cells measured by the mixed antiglobulin reaction (means 7.2–11.6 per cent). However, newborn infants had a significantly lower percentage of lymphocytes binding Fc of IgG (mean 10.4 per cent) as compared to adults (mean 17.2 per cent). No overall correlation between plasma Ig levels and Ig-bearing lymphocytes was found but a single infant with increased plasma IgA also showed the highest level of Ig-bearing lymphocytes among all infants.

Key words: Lymphocyte markers; B-cells; T-cells; newborn infants.

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Lymphocyte surface markers such as surface immunoglobulin, receptors for immunoglobulin and for complement factors and receptors for sheep erythrocytes are valuable tools in the identification of lymphocyte subpopulations in man (for review see *Hallberg* 1975).

Various investigators have by now established the normal levels in adults of lymphocytes bearing such markers whereas children and infants have been less extensively studied. In the present investigation, peripheral blood lymphocytes from newborn term and preterm infants were investigated by rosetting techniques for the presence of surface immunoglobulin, binding of Fc of IgG and binding of sheep erythrocytes. The results are compared with those obtained with lymphocytes from normal adult donors.

MATERIAL AND METHODS

Newborn Infants

Thirteen newborn, preterm infants (gestational age 33–37 weeks) and 7 newborn, term infants (gestational age 38–42 weeks) were investigated. The birth weight of all but one of the infants was appropriate for gestational age (AGA); one term infant was small for gestational age (SGA). None of the infants showed signs of infection or had received blood transfusions before testing. The age of the infants at time of testing is shown in Table 1. All the infants were hospitalized at the Department of Pediatrics, Malmö General Hospital. Among the preterm infants 7 were hospitalized just because of the prematurity whereas 3 infants suffered from hyperbilirubinaemia, 2 infants had asphyxia and one had signs of cerebral damage. In the group of term infants the patients were hospitalized because of hyperbilirubinaemia (2 patients), asphyxia (2), pulmonary atelectasis (1), cleft palate (1) or low birth-weight (1). Before collecting blood from the infants their parents were asked for the allowance to do so.

The study was supported by grants from the *Danish Medical Research Council* the *Danish Foundation for the Advancement of Medical Science* and in part by *F L. Smidth & Co A/S's Jubilæumsfond* I am grateful to *Lidy Broersma* and *Else Marie Nicolaisen* for excellent technical assistance.

REFERENCES

1 *Alford R H* Metal cation requirements for phytohemagglutinin-induced transformation of human peripheral blood lymphocytes. J Immunol. *104* 698–703 1970

2 *Augustinsson K B* Arylesterases. J Histochem. Cytochem. *12* 744–747 1964

3 *Austen K F & Becker E. L.* Biochemistry of the acute allergic reactions, Blackwell Scientific Publications Oxford 1971

4 *Bartfeld H & Atoynatan T* N acetylcysteine inactivates migration inhibitory factor and delayed hypersensitivity reactions. Nature N Biol. *231* 157–159 1971

5 *Bendtzen K* Affinity chromatography A new technique for partial purification of human leucocyte migration inhibitory factor J Immunol Methods *11* 147–151 1976.

6. *Bendtzen K* Inhibition of human leukocyte migration inhibitory factor (LIF) by a L fucose. Acta Allergol. *30* 327–338, 1975

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13 *Remold H G* The enhancement of MIF activity by inhibition of macrophage associated esterases. J Immunol. *112* 1571–1577 1974

14 *Rocklin R E. Remold H G & David J R.* Characterisation of human migration inhibitory factor (MIF) from antigen-stimulated lymphocytes Cell Immunol *5* 436–445 1972

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LYMPHOCYTE MARKERS IN NEWBORN INFANTS

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Hallberg, T & Hallberg, A. Lymphocyte markers in newborn infants Acta path. microbiol. scand. Sect. C, 84 477–484 1976.

Purified peripheral blood lymphocytes from 20 newborn infants and 12 adults have been studied for the presence of surface markers. Adults and infants did not differ in the percentage of sheep RBC-binding cells (means 58–65 per cent) or in Ig-bearing cells measured by the mixed antiglobulin reaction (means 7.2 11.6 per cent) However newborn infants had a significantly lower percentage of lymphocytes binding Fc of IgG (mean 10.4 per cent) as compared to adults (mean 17.2 per cent) No overall correlation between plasma Ig levels and Ig-bearing lymphocytes was found but a single infant with increased plasma IgA also showed the highest level of Ig-bearing lymphocytes among all infants

Key words Lymphocyte markers B-cells, T-cells newborn infants.

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Lymphocyte surface markers such as surface immunoglobulin, receptors for immunoglobulin and for complement factors and receptors for sheep erythrocytes are valuable tools in the identification of lymphocyte subpopulations in man (for review see *Hallberg* 1975)

Various investigators have by now established the normal levels in adults of lymphocytes bearing such markers whereas children and infants have been less extensively studied. In the present investigation, peripheral blood lymphocytes from newborn term and preterm infants were investigated by rosetting techniques for the presence of surface immunoglobulin, binding of Fc of IgG and binding of sheep erythrocytes. The results are compared with those obtained with lymphocytes from normal adult donors.

MATERIAL AND METHODS

Newborn Infants

Thirteen newborn, preterm infants (gestational age 33 37 weeks) and 7 newborn, term infants (gestational age 38–42 weeks) were investigated. The birth weight of all but one of the infants was appropriate for gestational age (AGA) one term infant was small for gestational age (SGA) None of the infants showed signs of infection or had received blood transfusions before testing The age of the infants at time of testing is shown in Table 1 All the infants were hospitalized at the Department of Pediatrics, Malmö General Hospital. Among the preterm infants 7 were hospitalized just because of the prematurity whereas 3 infants suffered from hyperbilirubinaemia, 2 infants had asphyxia and one had signs of cerebral damage. In the group of term infants the patients were hospitalized because of hyperbilirubinaemia (2 patients) asphyxia (2) pulmonary atelectasis (1) cleft palate (1) or low birth-weight (1) Before collecting blood from the infants their parents were asked for the allowance to do so.

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REFERENCES

1. *Alford R. H.* Metal cation requirements for phytohemagglutinin induced transformation of human peripheral blood lymphocytes. J. Immunol. *104* 698–703 1970
2. *Augustinsson K. B.* Arylesterases. J. Histochem. Cytochem. *12* 744–747 1964
3. *Austen K. F. & Becker E. L.* Biochemistry of the acute allergic reactions, Blackwell Scientific Publications Oxford 1971
4. *Bartfeld H. & Atoynatan T.* N acetylcysteine inactivates migration inhibitory factor and delayed hypersensitivity reactions. Nature N Biol *231* 157–159 1971
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9. *David J. R. & Becker E. L.* Inability to block the activity of guinea pig migration inhibitory factor with di isopropyl phosphorofluoridate. Eur J Immunol. *4* 287–289 1974
10. *Havemann K. Horvat M. Sodomann C. P. Havemann K. & Bürger S.* Protease activity as a possible mechanism of migration inhibitory factor. Eur J Immunol *2* 97–99 1972.
11. *Pick E. & Manheimer S.* The mechanism of action of soluble lymphocytic mediators. II Modification of macrophage migration and migration inhibitory factor action by drugs, enzymes and cationic environment. Cell Immunol. *11* 30–46 1974
12. *Polgar L. & Bender M. L.* A new enzyme containing a synthetically formed active site. Thiol-subtilisin. J. Am. Chem. Soc. *88 13* 3153–3154 1966
13. *Remold H. G.* The enhancement of MIF activity by inhibition of macrophage associated esterases. J Immunol *112* 1571–1577 1974
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TABLE 2. *Sheep RBC-binding Lymphocytes in Newborn Infants and Adults*

Individuals	N	Sheep RBC-binding lymphocytes (mean ± SE) Per cent	Absolute number per mm
Preterm infants	13	62.6 ± 4.2	3[illegible]63 ± 419
Term infants	7	63.3 ± 4.2	4650 ± 801
All infants	20	63.3 ± 3.1	3748 ± 407
Adults	12	58.1 ± 2.3	ND

ND not done

TABLE 3. *Ig-bearing Lymphocytes in Newborn Infants and Adults*

Individuals	N	Ig-bearing lymphocytes (mean ± SE) Per cent	Absolute number per mm
Preterm infants	11	10.7 ± 1.2	564 ± 79
Term infants	7	7.2 ± 1.6	519 ± 165
All infants	18	9.3 ± 1.0	546 ± 77
Adults	12	11.6 ± 1.6	ND

ND not done.

TABLE 4. *Fc-receptor-bearing Lymphocytes in Newborn Infants and Adults*

Individuals	N	Fc-receptor-bearing lymphocytes (mean ± SE) Per cent	Absolute numbers per mm^3
Preterm infants	12	12.4 ± 1.8	649 ± 125
Term infants	6	7.5 ± 1.4	543 ± 165
All infants	18	10.6 ± 1.4	613 ± 98
Adults	12	17.2 ± 2.4	ND

ND not done

Additional Tests

In all infants total as well as differential counts of white blood cells were performed. The level of plasma IgG, IgA and IgM was determined by electroimmuno assay (*Laurell* 1972).

RESULTS

The outcome of the rosetting tests in infants and adults is shown in Tables 2–4.

In Table 2 the results in the sheep RBC rosetting test (T cell marker) are shown. Adults and infants had 58–65 per cent sheep RBC-binding lymphocytes. The observed differences between the groups were not significant as judged by Student's t-test ($p>0.05$). Nor was there any significant difference in absolute number of rosetting lymphocytes between preterm and term infants.

The mixed antiglobulin reaction, visualizing Ig-bearing lymphocytes, is accounted for in Table 3. Adults and infants had 11.6–7.2 per cent Ig-bearing lymphocytes, the lowest values being found in the group of term infants. Again, no significant differences were seen between the groups.

As shown in Table 4, all infants had a mean of 10.6 per cent Fc rosetting lymphocytes, thereby differing significantly ($p<$

TABLE 1 *Age Distribution of Newborn Infants Tested for Lymphocyte Markers*

Infants	Total number	Boys	Girls	Age (days) at time of testing			
				1–3	4–7	8–15	16–30
Preterm	13	5	8	5	3	3	2
Term	7	5	2	4	1	1	1
Total number	20	10	10	9	4	4	3

Normal Donors

were healthy adult people working in the laboratory

Preparation of Lymphocytes for Testing

Approximately 2 ml of blood was collected in heparin from a scalp or cubital vein of the infant. Processing of the blood included sedimentation of red cells with methyl cellulose, magnetic removal of phagocytes after ingestion of iron particles and a final purification of the lymphocytes by centrifugation on Isopaque-Ficoll (for details see *Hallberg* 1974). For use in the mixed antiglobulin reaction the lymphocytes were treated with formaldehyde as described by *Hallberg et al.* 1974. Before testing the lymphocytes were made up to 2×10^6 per ml in diluent (phosphate buffered saline with 0.2 per cent bovine serum albumin).

The final lymphocyte suspension contained more than 95 per cent mononuclear cells, of which almost all excluded trypan blue dye. The suspension also contained some erythrocytes and platelets.

Blood samples from normal donors were treated as described above.

Rosette Forming Reactions

In all tests 1 drop of lymphocytes (2×10^6 per ml) and 1 drop of indicator red cells (0.8 per cent) were spun together in 7×50 mm siliconed glass tubes at $200 \times G$ for 5 minutes.

After centrifugation the cells were mounted with toluidine blue dye, and the number of rosettes, (i.e. lymphocytes binding at least 4 indicator cells) was counted among 200–300 lymphocytes. Each test was performed in quadruplicate.

Sheep Red Cell Rosetting Reaction

Lymphocytes and indicator cells were spun at room temperature and kept at +4 C over night before mounting.

Fc rosetting Reaction

Lymphocyte receptors for IgG were demonstrated by a rosetting reaction using antibody sensitized ox red cells as the indicator cells (*Hallberg et al.* 1973). Ox blood was obtained in Alsever's solution from a slaughter house. Differences in agglutinability of red cells between individual oxen (*Uhlenbruck et al.* 1967) may affect the rosetting reaction (*Hallberg*, unpublished data). However this difficulty was overcome by treating the red cells with neuraminidase as follows.

One ml of a ? per cent suspension of well washed ox red cells was spun in a glass tube and the supernatant discharged. The packed red cells were mixed with 12.5 IU of neuraminidase (Behringwerke preparation from Vibrio cholerae) in 0.5 ml of saline with 1 mg/ml of $CaCl_2$. The tube was incubated at 37 C for 30 minutes, the cells washed 3 times and resuspended in saline. These neuraminidase-treated ox RBC were sensitized with a pooled rabbit anti-ox red cell antiserum (K78/79) diluted 1:32, *i.e.* a dose just below the agglutinating capacity of the antiserum (for details see *Hallberg et al* 1973). For preparation of rosettes lymphocytes and indicator cells were spun in the cold and kept in ice for 1–2 hours before mounting. Two of the 20 infants could not be tested for Fc-binding lymphocytes because of shortage of lymphocytes.

Mixed Antiglobulin Reaction for Detection of Lymphocyte Surface Immunoglobulin

This test was performed on formalinized lymphocytes as described by *Hallberg et al.* (1974). The antiglobulin reagent was a rabbit antiserum (K 8) raised against DEAE-cellulose-purified human IgG and reactive with both heavy and light chains. Indicator cells were ox red cells sensitized with antibodies from human infectious mononucleosis serum. Controls in the mixed antiglobulin reaction included lymphocytes treated with normal rabbit serum instead of antiglobulin reagent, as well as tests with non-sensitized indicator ox red cells. As a further control of specificity it was checked that all positive reactions could be inhibited by soluble human IgG. Two of the 20 infants could not be tested for Ig-bearing lymphocytes because of shortage of lymphocytes.

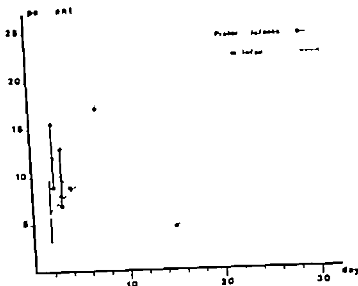


Fig 2 Per cent Fc-receptor bearing lymphocytes in the blood of term and preterm infants as related to age of infant at time of testing. Each dot or square marks the result in one individual. Preterm infants, gestational age 33–37 weeks. Term infants: gestational age 38–42 weeks

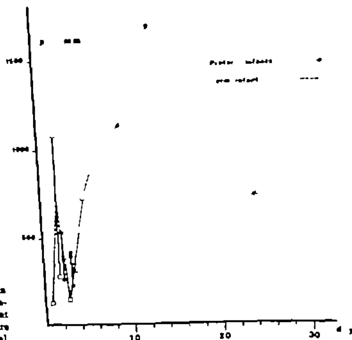


Fig 3 Number per mm³ of Fc receptor bearing lymphocytes in the blood of term and preterm infants as related to age of infant at time of testing. Each dot or square marks the result in one individual.

two (preterm) infants who had 0.5 and 0.8 g/l, respectively The first of these two infants had a high level of Ig-bearing lymphocytes (1330 per mm) whereas the second child had a normal or low level (460 per mm)

DISCUSSION

In the present investigation newborn infants and adults were found not to differ significantly as regards the percental proportion in peripheral blood of lymphocytes bearing re

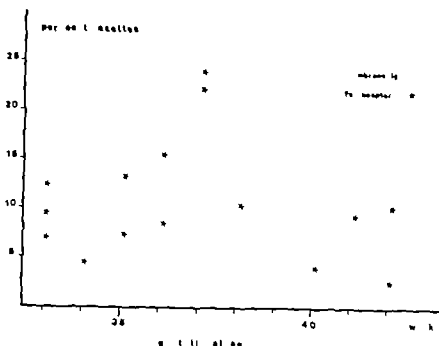


Fig 1 Per cent Ig-bearing and Fc receptor bearing lymphocytes in the blood of newborn infants as related to gestational age Each symbol marks the result in one individual.

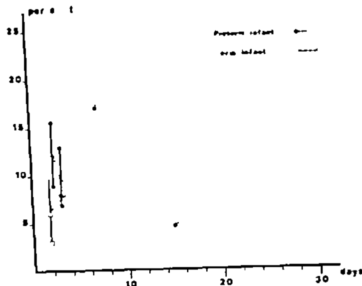
0 05) from adults (mean 17 2 per cent) Among the infants, term babies had a significantly lower proportion of Fc rosetting lymphocytes as compared to preterm babies (7 1 and 12 4 per cent, respectively P<0 05) In contrast the absolute numbers of Fc rosetting lymphocytes did not differ significantly between infants of various gestational age.

It may be asked to what extent the data in the rosetting tests were influenced a) by variations in gestational age and b) by variations in age of infants when tested. The variations in the mixed antiglobulin reaction and the Fc rosetting reaction as related to gestational age are shown in Fig 1 The tendency is the same in both tests *i.e.* a peak around week 37 However data are based on a small number of individuals. In the sheep RBC rosetting test gestational age did not influence the results. In the group of term infants one individual was small for gestational age (SGA) This infant had a lower percentage of Ig bearing lymphocytes (1.8 per cent) and a higher percentage of sheep RBC binding lymphocytes (78.4 per cent) than the remaining term infants. This child was not tested for Fc binding lymphocytes.

The percentage and total number of Fc binding lymphocytes as related to the age of the newborn infants at time of testing is shown in Figs. 2 and 3 Preterm infants showed peak values in this test during the second week of life. The same tendency of increase in Fc rosettes with age was seen for term infants, but none of these was older than 6 days at time of testing Again it must be realized that rather few infants have been tested. In the mixed antiglobulin reaction and the sheep RBC rosetting tests no consistent patterns were seen as related to the age of the infants.

The range of the plasma Ig levels of the infants is shown in Table 5 No correlation between number of Ig-bearing lymphocytes and plasma IgG level was seen As expected, preterm infants had a lower concentration of plasma IgG as compared to term infants. In contrast to IgG the levels of IgA and IgM probably mainly reflects the Ig production of the infant itself IgA was low and normal in all but one (term) infant who had 1.3 g/l. This infant also had the highest number of Ig bearing lymphocytes recorded among all infants investigated (1420 per mm) Plasma IgM was normal (0.3 g/l or less) in all but

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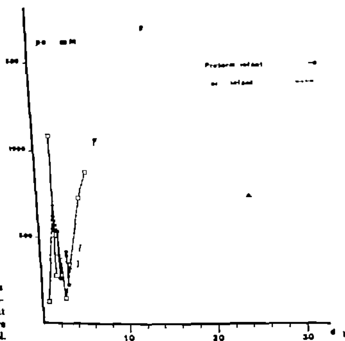


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two (preterm) infants who had 0.5 and 0.8 g/l, respectively. The first of these two infants had a high level of Ig-bearing lymphocytes (1330 per mm³), whereas the second child had a normal or low level (460 per mm³).

DISCUSSION

In the present investigation newborn infants and adults were found not to differ significantly as regards the percental proportion in peripheral blood of lymphocytes bearing re-

TABLE 5 *Plasma Immunoglobulin Levels in the Present Material of 20 Newborn Infants*

Infants	*N*	Level of plasma Ig in grams/litre (range)		
		IgG	IgA	IgM
Preterm	13	2– 7	0 2–0 4	0.04–0.8
Term	7	3–12	0 4–1.3	0 06–0.23

ceptors for sheep RBC (T cell marker) or membrane-associated immunoglobulin. On the other hand newborn infants had a significantly lower percentage of lymphocytes binding IgG as indicated in an opsonic rosetting test.

The proportions of T and B cells in newborns have earlier mainly been studied in cord blood whereas venous blood was used here. In accordance with *Eife et al* 1974 *Ferguson et al* 1974 and *Lalla* 1975 we found equal proportions of sheep RBC-binding lymphocytes in newborn infants and adults whereas others (*Campbell et al.* 1974 *Matsaniotis et al* 1974 *Smith et al* 1974 *Diaz Jouanen et al* 1975 *Fleisher et al* 1975) have reported lower proportions of sheep RBC-binding lymphocytes in newborns and young children as compared to adults Even so the absolute numbers of sheep RBC-binding lymphocytes in newborns have equalled or exceeded those in adults due to the high numbers of lymphocytes in the blood of newborn infants. Besides differences in technology gestational age as well as age of the infants at time of testing might influence the results, although no such age-dependences were seen in the present tests for sheep RBC rosettes. However *Ferguson et al* (1974) did find a lowered percentage of sheep RBC-rosettes in children small for gestational age as compared to children appropriate for gestational age and adults.

Membrane-associated immunoglobulin is considered to be the main marker for B lymphocytes. Levels of Ig bearing lymphocytes in healthy adults vary widely between different investigators (see *Warner* 1974) and values between 5 and 30 per cent have been reported In the present investigation a poly specific anti human immunoglobulin anti serum raised against human IgG but not absorbed to be class specific revealed similar levels of Ig-bearing lymphocytes in newborns and in adults. In earlier investigations newborns have been found to have equal (*Ugazio et al.* 1974 *Diaz Jouanen et al* 1975) or higher (*Papamichail et al* 1971 *Froland & Natvig* 1972 *Campbell et al* 1974 *Moscatelli et al* 1973) proportions of Ig-bearing lymphocytes than adults Again these differences are probably mainly due to differences in technology No overall correlation between plasma Ig levels and proportion of Ig-bearing lymphocytes have been found. In contrast, it is known that IgD in spite of its low plasma concentration, occurs on a majority of Ig bearing lymphocytes partly together with IgM (*Rowe et al* 1973 *Fu et al* 1975) In the present investigation no attempts to identify the class of the membrane-bound Ig on the lymphocytes were undertaken A salient observation was that the two highest numbers of Ig bearing lymphocytes occurred in two infants with increased levels of plasma IgA and IgM ,respectively (for reference values of plasma Ig levels see *Johansson & Berg* 1967)

It may be asked whether Ig-bearing lymphocytes are indeed Ig producing cells, and recent investigations (*Winchester et al.* 1975 *Lobo et al* 1975) indicate that a certain proportion of Ig bearing lymphocytes have picked up their immunoglobulin from without. When special precautions are taken to exclude these cells a mean of about 10 per cent Ig bearing lymphocytes are found in the peripheral blood of healthy adults. In the present investigation healthy adults had a mean of 11 6 per cent Ig-bearing lymphocytes. We

cannot exclude that some Fc binding lymphocytes may be included among the Ig-bearing cells and if this is so, it might explain part of the co-variation between Ig-bearing and Fc binding lymphocytes in the newborns (Fig. 1) However in contrast to Ig-bearing lymphocytes, the level of Fc-binding lymphocytes was significantly lower in newborn term or preterm infants as compared to adults. This low level of Fc-binding lymphocytes agrees well with the finding of *Campbell et al.* (1974) of a low level of K-cell activity in lymphocytes from cord blood since this K cell activity is dependent upon cells with receptors for Fc of IgG On the other hand, the level of Fc rosetting lymphocytes possibly varies with the gestational age and the age of the infant at time of testing (cf. Figs. 1 ? and 3) This prediction is based on a fairly small number of observations in different individuals and investigations are now under way to study the development of lymphocyte surface markers with increasing age in individual infants.

Excellent technical assistance was given by Mrs. *Ulla Regnér* and Miss *Annie Andersson*. Determinations of plasma Ig levels were performed at the Department of Clinical Chemistry Malmö General Hospital (Head Professor *C.-B. Laurell*) This investigation was supported by grants from the *Medical Faculty University of Lund* and from *Kungliga Fysiografiska Sällskapet Lund*.

REFERENCES

Campbell A C Waller C Wood J Aynsley-Green A & Y V Lymphocyte subpopulations in the blood of newborn infants. Clin. exp. Immunol 18 469–482, 1974

Diaz-Jouanen, E. Williams J R C & Strickland R G Age-related changes in T and B-cells Lancet 688–689 1975

Ed R F Ed O A gust C S Kuhr W L & Stachr-Johansen K Lymphotoxin production and blast cell transformation by cord blood lymphocytes dissociated lymphocyte function in newborn infants Cell. Immunol. *14* 435–442, 1974

Ferguson, A C Lawlor J G J Neumann Ch. G Oh W & Stiehm E R. Decreased rosette-forming lymphocytes in malnutrition and intrauterine growth retardation. J Pediatrics *85*: 717 723 1974

Fleisher T A, Luckasen J R Sabad A, Gehrz R C & Kersey J H.: T and B lymphocyte subpopulations in children. Pediatrics 55: 162 165 1975

Fröland S S & Natvig, J B. Lymphocytes with membrane-bound immunoglobulin (B-lymphocytes) in newborn babies. Clin. exp. Immunol. *11* 495–505 1972.

F S M Winchester R J & Kunkel, H G. Similar idiotypic specificity for the membrane IgD and IgM of human B lymphocytes. J Immunol. *114* 250–252, 1975

Hallberg T In vitro cytotoxicity of human lymphocytes Reduction of the lymphocyte cytotoxicity induced by antibodies or phytohaemagglutinin by removing immune-complex-binding cells. Scand. J Immunol. 3 645–654 1974

Hallberg, T Human lymphocyte subpopulations. Acta path. microbiol. scand. Sect. C, suppl. 250 1975 p. 1 54

Hallberg, T G ner B. W & Coombs R. R. A Opsonic adherence of sensitized ox red cell to human lymphocytes as measured by rosette formation. Int. Arch Allergy *44* 500–513 1973

Hallberg, T Haegert D Clein G P Coombs R. R. A Feinstein A & Gurner B. W. Observations on the mixed antiglobulin reaction as a test for immunoglobulin-bearing lymphocytes in normal persons and in patients with chronic lymphatic leukaemia. J Immunol. Methods *4* 317 332, 1974

Johansson S G O & Berg, T Immunoglobulin levels in healthy children. Acta Paediat. Scand. *56* 572–579 1967

Lalla M Proliferation of blood leucocytes in the neonatal period. Acta haemat. 53 129–137 1975.

Laurell, C.-B Electroimmuno assay Scand. J clin. Lab. Invest. 29 suppl. 124 21 37 1972.

Lobo P I Westervelt F B. & Horwitz, D A Identification of two populations of immunoglobulin-bearing lymphocytes in man. J Immunol *114* 116–119 1975

Matsaniotis, N Mantalenaki-Asfi C Apostolou M & Liakopoulou P Age dependence of T cells. Brit. Med. J 3 743 1974

Moscatelli, P Bricarelli, F D & d Barbieri, A.: Immunoglobulins on the surface of lymphocytes, their distribution in cord blood, newborns and infants affected by transient hypogammaglobulinaemia Helv paediat. Acta 28 553–558, 1973

Papamichail M Brown J C & Holborow E. J.: Immunoglobulins on the surface of human lymphocytes. Lancet ii: 850–852, 1971

Rowe D S Hug, K Forni L. & Pernis B Immunoglobulin D as a lymphocyte receptor J exp Med. *138* 965–972 1973

Smith M A Evans J & Steel C M Age-related variation in proportion of circulating T cells. Lancet *ii* 922–924 1974

Ugazio A G Marcioni A F Astaldi A Jr & Burgio G R Peripheral blood B lymphocytes in infancy and childhood. Acta Paediat. Scand. *63* 205–208 1974

Uhlenbruck G Seaman G V F & Coombs R R A Factors influencing the agglutinability of red cells. III Physicochemical studies on ox red cells of different classes of agglutinability Vox Sang *12* 420–428, 1967

Warner N L. Membrane immunoglobulins and antigen receptors on B and T lymphocytes. Adv Immunol. *19* 67–216 1974

Winchester R J Fu S M Hoffman T & Kunkel, H G IgG on lymphocyte surfaces technical problems and the significance of a third cell population. J Immunol. *114* 1210–1212 1975

Acta path. microbiol. scand. Sect. C, 84: 485–488, 1976

LEUCOCYTE MIGRATION INHIBITORY ACTIVITY (LMIA) ON CONCANAVALIN A (CON A) STIMULATED HUMAN LYMPHOCYTES

Comparison of Leucocyte Migration Capillary Technique (LMCT) Leucocyte Migration Agarose Technique (LMAT) and Leucocyte Migration Fibrinolysis Technique (LMFT)

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Laboratory of Clinical Immunology, Medical Department TA,
Rigshospitalet University Hospital, Copenhagen, Denmark

Coeugniet, E. & Bendixen, G. Leucocyte migration inhibitory activity (LMIA) of Concanavalin A (Con-A) stimulated human lymphocytes. Comparison of leucocyte migration capillary technique (LMCT), leucocyte migration agarose technique (LMAT) and leucocyte migration fibrinolysis technique (LMFT). Acta path. microbiol. scand. Sect. C, 84: 485–488, 1976.

Leucocyte migration and migration inhibition in fibrin medium may reveal new aspects of lymphokine activity associated with immunological *inflammation*. Leucocyte migration fibrinolysis technique is compared with the leucocyte migration capillary technique and the leucocyte migration agarose technique. In the present model experiment the three methods gave comparable results. The leucocyte migration fibrinolysis technique involves a new principle for detection of lymphokines and can probably be developed to give more exact information about the interrelationship between thrombosis, fibrinolysis and lymphokines.

Key words: Concanavalin A; fibrinolysis; leucocyte migration; lymphokines; migration inhibition.

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Interaction between normal lymphocytes and mitogens, e.g. Concanavalin A (Con A) or between sensitized lymphocytes and specific antigen *in vitro* results in the generation of lymphocyte-derived, soluble factors, commonly known as lymphokines, with a variety of biological activities. One of these factors exert leucocyte migration inhibitory activity (LMIA) which, if measured in the proper *in vitro* system, can be used as a quantitative assay for lymphokines. Two methods are by now well-standardized for determination of LMIA: the leucocyte migration capillary technique (LMCT) originally described by *Søborg & Bendixen* (1, 2) and the leucocyte migration agarose technique described by *Clausen* (2, 3). *In vivo* and *in vitro* however cell migration, cell migration inhibition and cell ability to penetrate tissue is influ-

enced by multiple other factors among which release of proteolytic activity (4, 5 6 7 8 9 11) is considered of major importance The present report describes an *in vitro* system for analysis of this aspect of the inflammatory process and its relation to lymphokines. The system measures the ability of leucocytes by migration to *penetrate* a fibrin mass. This leucocyte migration fibrinolysis technique (LMFT) registers a combination of fibrinolytic and migratory activity of leucocytes (5) In the present study, the results obtained with the same cell populations using LMCT LMAT and LMFT in parallel assays are compared, measuring human leucocyte migration responses to LMIA-containing human lymphocyte culture supernatants.

MATERIAL AND METHODS

Preparation of LMIA-containing Culture Supernatants

Venous blood from healthy adult donors was stabilized with heparin 25 i.u./ml and diluted with an equal volume of Hanks balanced salt solution (HBSS) The mononuclear cells (lymphocytes and monocytes) were separated by Ficoll gradient centrifugation (2) washed in HBSS and suspended in tissue culture medium 199 (Difco, Michigan, USA) (TC 199) containing penicillin 67 i.u./ml and streptomycin 67 μg/ml 250 μg Con-A (Pharmacia Uppsala Sweden) in 50 μl TC 199 was added to 3 ml cell suspension. The final cell concentration was 2.5×10^6 cells/ml. The mononuclear cell suspension was incubated at 37 C in 2 per cent CO_2 in atmospheric, water-saturated air After 22 h the cultures were terminated by centrifugation at $670 \times g$ for 10 min and 50 μl TC 199 was added to the cell-free supernatant In each test a supernatant was prepared as parallel control in the following way Before incubation, 50 μl TC 199 without Con-A was added to a 3 ml cell suspension identical with the sample. After incubation and centrifugation, the cell free supernatant was reconstituted with 50 μl TC 199 containing 250 μg Con A.

Removal of Con-A and TC 199 Compounds from Culture Supernatants

Removal of TC 199 compounds K 16/20 Sephadex G 50 columns (Pharmacia) volume bed 20 ml were equilibrated with ammonium-bicarbonate buffer (50 mMol/l adjusted at pH 7.0 with acetic acid. 6 ml cell free Con-A preincubated and control culture supernatants were applied on parallel columns at room temperature. The compounds of TC 199 were removed from the supernatants by passage through the columns, the procedure involving a 1.5 times dilution.

Removal of Con A K 16/20 Sephadex G-100 columns volume bed 16 ml were equilibrated with ammoniumbicarbonate buffer at pH 7 0 as indicated above. Con A was removed from Con-A pre-incubated and control supernatants by conjugation to Sephadex (2) the supernatants hereby being diluted another three times. The initial supernatant volume of 6 ml was thus diluted into 9 ml by the Sephadex G 50 passage and to 27 ml by the Sephadex G-100 passage. The mitogen free (2) supernatants were passed through Millipore® filters (0 45 μm pore size) and lyophilized Supernatants from 24 persons were prepared in this way and collected in 4 pools, each containing equal quantities of stimulated and control supernatants from 6 persons.

The LMIA of the lyophilized supernatant pools was tested after redissolution in TC 199 with 10 per cent horse serum at a concentration corresponding to the initial culture supernatant.

Techniques for Measuring LMIA

I Leucocyte migration capillary technique (LMCT) LMCT was performed as described by *Bendixen & Søborg* (1) and *Bendixen et al.* (2) and modified according to *Marni et al* (10) Briefly leucocyterich plasma obtained after dextran sedimentation of blood stabilized with heparin was centrifuged at $225 \times g$ for 5 min. The leucocytes were washed in HBSS 3 times and, after resuspension in TC 199 with 10 per cent horse serum they were collected in 20 μl capillary tubes with an internal diameter of 0 6 mm, each tube containing 3×10^6 leucocytes. After sealing one end in a flame and centrifugation at $900 \times g$ for 10 min each capillary tube was cut just below the cell fluid interface and placed in an 0.5 ml culture chamber containing redissolved Con-A preincubated or control culture supernatant All samples were examined in quadruplicate. The leucocyte migration areas around the capillary tube openings were measured in a projection microscope after 22 h at 37 C The migration index (MI) was calculated as the ratio between the migration area of leucocytes in Con A pre-incubated and control supernatant in the following way

$$MI = \frac{\text{Mean of migration areas in Con-A preincubated supernatant}}{\text{Mean of migration areas in control supernatant}}$$

A MI below 1 0 indicates inhibition, a MI above unity stimulation of the migration.

TABLE 1 *Demonstration of Leucocyte Migration Inhibitory Activity (LMIA) by LMCT, LMAT and LMFT*

Indicator leucocyte population nr	MI LMCT	MI LMAT	MI LMFT
1	0.56	0.68	0.63
2	0.76	0.85	0.81
3	0.80	0.85	0.85
4	0.63	0.72	0.70
5	0.62	0.74	0.76
6	0.76	0.83	0.85
7	0.72	0.74	0.78
8	0.70	0.85	0.80
9	0.57	0.68	0.66
10	0.70	0.75	0.78
Mean of MI ± SD	0.68 ± 0.08	0.77 ± 0.07	0.76 ± 0.07

LMCT leucocyte migration capillary technique. LMAT leucocyte migration agarose technique. LMFT leucocyte migration fibrinolysis technique. MI migration index. SD standard deviation

2 Leucocyte migration agarose technique (LMAT) The technique was performed as described by *Clausen* (3). In brief 20 × 10⁶ leucocytes were suspended in 100 µl Con-A pre-incubated or control culture supernatant. After 1 h of incubation at 37 C, the cells were placed in wells in agarose and the migration under agarose was determined by measuring the migration areas after 22 h of incubation at 37 C in 2 per cent CO_2 and water-saturated air. The LMIA was calculated as MI according to the formula indicated above.

3 Leucocyte migration fibrinolysis technique (LMFT) The essential features of this technique has been described elsewhere (5). Fibrin plates were prepared in the following way: Human lyophilized fibrinogen (Kabi, Stockholm, Sweden) was dissolved 1 g/100 ml in distilled water. 2 ml of the solution was mixed with 0.9 ml ten times concentrated TC 199, 1 ml horse serum, 6.1 ml distilled water, 50 µl 10 per cent $NaHCO_3$ and penicillin and streptomycin at final concentrations of 67 i.u. and 67 µg/ml, respectively.

From this mixture, aliquots of 7.5 ml were poured into plastic petri dishes with a diameter of 85 mm (Nunc, Roskilde, Denmark) and clotted at room temperature with 0.3 ml thrombin solution (50 NIH/ml). After 30 min at room temperature, 7 µl aliquots of leucocytes (10 × 10⁶ cells per 100 µl) suspended in Con-A-incubated or control supernatant were carefully applied onto each plate by micropippettes (H. Pedersen, Copenhagen, Denmark) in order that spheric drops might be placed on fibrin surface areas of identical size. The fibrin plates were incubated for 22 h at 37 C in water saturated atmosphere containing 2 per cent CO_2.

Calculations

All samples were examined in quadruplicate and ten different indicator leucocyte populations were tested with Con-A preincubated and control supernatant pools as described above. Wilcoxon test was used for estimation of significant difference.

RESULTS

The results are presented in Table 1. The LMIA of the Con A stimulated mononuclear cell cultures examined is demonstrated equally well by all three techniques. No significant differences between means of MI were observed, as calculated on the basis of LMCT, LMAT and LMFT.

DISCUSSION

The types of migration registered by LMCT, LMAT and LMFT are different. In LMCT (1, 2) the cells are migrating in liquid medium from the opening of the capillary tube along the bottom of a culture chamber. In LMAT the cells are migrating under the gel culture medium on a plastic surface (2, 3). In the LMFT the leucocytes are migrating

through the fibrin space and thus register the ability of leucocytes to penetrate a fibrin mass probably depending on a combination of fibrinolytic activity and migration activity (4, 5 6) It is of particular interest that excellent migration areas can be obtained in the LMFT by half the number of cells used in LMAT or by one tenth of the cells used in LMCT As indicated by *Bendixen et al* (2) the LMCT is unfit for measuring LMIA in indirect (two-step) assays, whereas the LMAT is recommendable for this purpose. The LMFT seems to be just as sensitive as the LMAT for measuring LMIA in the second step of two-step assays. The LMAT and the LMFT however, do not register the same combination of lymphokine activity since the fibrinolytic effect of granulocyte material and its modification by lymphokines is of importance for leucocyte migration in the fibrin mass (4 5) In the present experiment, the gross results of the migratory process in LMCT LMAT and LMFT were comparable but the LMFT can probably be further elaborated in order that supplementary information may be provided by parallel examination of migration cultures in which modulators of the fibrinolytic system are included

REFERENCES

1 *Bendixen G & Søborg, M* A leucocyte migration technique for in vitro detection of cellular (delayed type) hypersensitivity in man. Dan Med. Bull. *16* 1–6 1969

2 *Bendixen G Bendixen K., Clausen J Kjær M & Søborg, M* Human leucocyte migration inhibition In vitro techniques and applications. Scand. J Immunol. Supplementum IV 1976

3 *Clausen J* Tuberculin-induced migration inhibition of human peripheral leucocytes in agarose medium. Acta allergol. *26* 56–80 1971

4 *Coenguist E. & Bendixen G* Lymphokines and thrombosis. III Influence of active culture supernatants of Con A stimulated lymphocytes on fibrinolysis. Unpublished results.

5 *Coenguist E. & Bendixen G* Leucocyte migration fibrinolysis technique (LMFT) Description of a method. Biomedicine, 1976, in press.

6. *Coenguist E Søeberg B & Bendixen G* Human leucocyte response in agarose and fibrin medium to migration inhibitory activity from lymphocytes. Modification by aprotinin, tranexamic acid and phenylmethyl sulfonylfluoride. Unpublished results.

7 *Latner A Longstaff E & Pradhan K* Inhibition of malignant cell invasion *in vitro* by a proteinase inhibitor Brit. J of Cancer *27* 460–464 1973

8. *Latner A Longstaff E & Turner G* Anti tumor activity of aprotinin. Brit. J of Cancer *30* 60–67 1974

9 *Liedholm P Astedt B. & Kullander S* Passage of tranexamic acid (AMCA) to semen in man and its effect on the fibrinolytic activity and on migration of spermatozoa. Fertil and Steril *24* 517–520 1973

10 *Maini R N Rolfe L M Magrath J T & Dumonde D C* Standardization of the leucocyte migration test. Int. Arch. Allergy *45* 308–321 1973

11 *Saba, H Herion J Walter R & Roberts H* Effect of lysosomal cationic proteins from polymorphonuclear leucocytes upon the fibrinogen and the fibrinolysis system. Thrombos. Res. *7* 543–554 1975

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FREEZING OF RAT LYMPHOCYTES

III Freezing of Plaque-forming Cells and Restoration by Frozen-thawed Normal Cells of Antibody Production in Irradiated Rats

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Heim, E. Freezing of rat lymphocytes. III. Freezing of plaque-forming cells and restoration by frozen-thawed normal cells of antibody production in irradiated rats. Acta path. microbiol. scand. Sect. C, 84 489–494 1976.

The present investigation is an extension of earlier work with dimethyl sulphoxide (DMSO) protected frozen-thawed rat lymphocytes. In the present work it is shown that some 85–90 per cent of the haemolytic plaque-forming cells (PFC) survived the freeze-thaw process. Irradiated rats were restored with fresh and frozen-thawed cells and immunized against sheep red blood cells (SRBC) Evidence is presented that restrictive control of the PFC response by suppressor cells present in the spleen cell suspension is lost during the freeze-thaw process, giving higher number of PFC/spleen in recipients of frozen-thawed mixed spleen and lymph node cells than in rats receiving the corresponding fresh preparations.

Key words Rat lymphocytes freezing plaque-forming cells

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Low temperature preservation of rat lymphocytes has been studied in a series of experiments It has been found that cryoprotected spleen and lymph node cells responded *to non-specific mitogens after* thawing, and that frozen-thawed spleen cells responded better to phytohaemagglutinin (PHA) than did fresh spleen cells. This was probably caused by an inhibitory effect of the freezing procedure on a suppressor cell in the spleen (10) Moreover it has been found that the organ distribution of cryoprotected intravenously injected frozen-thawed lymphoid cells is principally the same as that applying to fresh cells (11)

The present paper deals with the ability of frozen-thawed rat lymphoid cells to produce antibodies against sheep red blood cells (SRBC) and to restore the immune response to SRBC in whole-body irradiated animals. The experiment shows that the antibody producing cells survive in high numbers, that they can restore the reactivity of irradiated rats, and that the spleen suppressor cells also in this experiment seem to be sensitive to the freezing procedure.

MATERIALS AND METHODS

Animals Inbred, locally grown hooded rats 9–12 weeks old were used. Rats of either sex were used in each experiment. Irradiated rats were always female.

Cell suspensions: A single cell suspension was

through the fibrin space and thus register the ability of leucocytes to penetrate a fibrin mass probably depending on a combination of fibrinolytic activity and migration activity (4, 5 6) It is of particular interest that excellent migration areas can be obtained in the LMFT by half the number of cells used in LMAT or by one tenth of the cells used in LMCT As indicated by *Bendixen et al* (2) the LMCT is unfit for measuring LMIA in indirect (two-step) assays, whereas the LMAT is recommendable for this purpose. The LMFT seems to be just as sensitive as the LMAT for measuring LMIA in the second step of two-step assays. The LMAT and the LMFT however do not register the same combination of lymphokine activity since the fibrinolytic effect of granulocyte material and its modification by lymphokines is of importance for leucocyte migration in the fibrin mass (4 5) In the present experiment, the gross results of the migratory process in LMCT LMAT and LMFT were comparable but the LMFT can probably be further elaborated in order that supplementary information may be provided by parallel examination of migration cultures in which modulators of the fibrinolytic system are included

REFERENCES

1 *Bendixen G & Søborg M* A leucocyte migration technique for in vitro detection of cellular (delayed type) hypersensitivity in man. Dan. Med. Bull. *16* 1–6, 1969

2 *Bendixen G Bendtzen K Clausen J Kjær M & Søborg M* Human leucocyte migration inhibition In vitro techniques and applications, Scand. J Immunol Supplementum IV 1976

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5 *Cosnguist E. & Bendixen G* Leucocyte migration fibrinolysis technique (LMFT) Description of a method. Biomedicine, 1976, in *press.*

6 *Cosnguist E Seeberg B & Bendixen G* Human leucocyte response in agarose and fibrin medium to migration inhibitory activity from lymphocytes. Modification by aprotinin tranexamic acid and phenylmethyl sulfonyl fluoride. Unpublished results.

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8 *Latner A., Longstaff E & Turner G* Antitumor activity of aprotinin. Brit. J of Cancer *30* 60–67 1974

9 *Liedholm P Åstedt B & Kullander S* Passage of tranexamic acid (AMCA) to semen in man and its effect on the fibrinolytic activity and on migration of spermatozoa. Fertil and Steril *24* 517 520 1973

10 *Maini R N Roffe L. M., Magrath J T & Dumonde D C* Standardization of the leucocyte migration test. Int. Arch. Allergy *45* 308–321 1973

11 *Saba H Herion J Walter R & Roberts H* Effect of lysosomal cationic proteins from polymorphonuclear leucocytes upon the fibrinogen and the fibrinolysis system. Thrombos. Res *7* 543–554 1975

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FREEZING OF RAT LYMPHOCYTES

III. Freezing of Plaque-forming Cells and Restoration by Frozen-thawed Normal Cells of Antibody Production in Irradiated Rats

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The present investigation is an extension of earlier work with dimethyl sulphoxide (DMSO) protected frozen-thawed rat lymphocytes. In the present work it is shown that some 85–90 per cent of the haemolytic plaque-forming cells (PFC) survived the freeze-thaw process. Irradiated rats were restored with fresh and frozen-thawed cells and immunized against sheep red blood cells (SRBC). Evidence is presented that a restricting control of the PFC response by suppressor cells present in the spleen cell suspension is lost during the freeze-thaw process, giving a higher number of PFC/spleen in recipients of frozen-thawed mixed spleen and lymph node cells than in rats receiving the corresponding fresh preparations.

Key words: Rat lymphocytes; freezing; plaque-forming cells.

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The present paper deals with the ability of frozen-thawed rat lymphoid cells to produce antibodies against sheep red blood cells (SRBC) and to restore the immune response to SRBC in whole body irradiated animals. The experiment shows that the antibody producing cells survive in high numbers, that they can restore the reactivity of irradiated rats, and that the spleen suppressor cells also in this experiment seem to be sensitive to the freezing procedure.

MATERIALS AND METHODS

Animals. Inbred, locally grown hooded rats 9–12 weeks old were used. Rats of either sex were used in each experiment. Irradiated rats were always female.

Cell suspensions. A single cell suspension was

prepared Spleen and/or lymph nodes (cervical brachial and mesenterial) were teased in medium RPMI 1640 with Hepes buffer (Gibco-Biocult Glasgow Scotland) and this medium was used throughout Cells were aspirated through a 25 G needle filtered through a nylon net and washed three times at 150 *g* for 8 min. All suspensions were again filtered and counted in an electronic cell counter before injection or use in the plaque assay

Freezing and thawing The cells were suspended in RPMI 1640 with 50 per cent fresh rat serum (FRS) in 2.5 ml samples at a concentration of 10^8 cells/ml. Another 2.5 ml medium with 20 per cent dimethyl sulphoxide (DMSO) was added gradually during 2.5 min the suspension injected into teflon/kapton bags and allowed to equilibrate for a further 7.5 min at room temperature. The bag was pressed between aluminium plates, and cooling took place in a Revco ultra low freezer operating at –90 C. The bag was placed above the poly styrene for 10 min, then in the bottom of the freezer for 10 min, and finally stored in liquid nitrogen for 10 min. The temperature was recorded by means of a thermocouple inserted into a control bag containing the same medium and cells the freezing curve was reproducible giving a freezing rate of 2 /min to 0 C and 6 /min to –60 C when the bag was transferred to –196 C. Crystallization of water ice took place between –9 and –12 elevating the temperature by 5–8 for some seconds. Due to the large surface of this bag, the freezing curve returned to the original slope within 30–40 sec. Cells have also been frozen at a fixed rate of –1 C (10) However as cell recoveries were similar the simpler procedure was used in the present experiment. Thawing was carried out in a waterbath at 37 C, and 45 ml RPMI 1640 with 10 per cent FRS were added at room temperature during 2 5 min. Finally the cells were centrifuged at 100 *g* for 5 min and resuspended.

Sheep red blood cells SRBC were stored in Alsever's solution at 4 C for up to three weeks before use. Erythrocytes were washed three times in normal saline and resuspended at the appropriate concentrations.

Irradiation The irradiations were performed by a Siemens X ray apparatus operated at 200 kV 20 mA and 0.5 mm Cu. Dose rate was 88 rad/min at a target distance of 40 cm.

Determination of plaque forming cells (PFC) The haemolytic plaque assay of *Jerne et al.* (14) was used Only direct PFC were determined. Petri dishes with a bottom layer of 1 4 per cent agarose (Indubiose A 37 L Industrie Biologique Française, Gennevilliers, France) in RPMI 1640 and a top layer of 0 7 per cent agarose were prepared 0 1 ml of 15 per cent SRBC was added to the top layer immediately before the addition of the lymphocytes. After incubation for 1 hour at 37 C, the dishes were flooded with 10 per cent guinea pig serum in phosphate buffered saline and again incubated for 30 min. Four dishes were prepared from each suspension and the plaque counts were taken as the value representing the plaque forming activity of the cell suspension. PFC per 5 10^8 cells and per spleen were calculated on the basis of this value The mean response and standard deviations were calculated on log transformed data (19)

Haemagglutinin titration Rats were bled from their tails and separated sera were stored at –26 C. After inactivation of complement for 30 min at 56 C, haemagglutinating antibody was titrated in two-fold dilutions in round-bottom microtrays (Linbro Chemicals, New Haven Conn.)

Statistics Student's t test was used to determine the significance of differences between means.

EXPERIMENTS AND RESULTS

Recovery Approximately 60–70 per cent of the cells injected into the bag were recovered after washing and resuspension of the frozen thawed cells. 8–10 per cent of the recovered cells did not exclude tryphan blue, as compared with 3–5 per cent before freezing

Freezing of plaque forming cells (PFC) This experiment was performed to determine the survival of antibody producing lymphocytes after freeze-thawing Rats were immunized intraperitoneally (i p) with 1 ml 50 per cent SRBC and their spleens were removed four days later as this gave the maximal number of PFC/spleen. The number of PFC in the suspensions of fresh and frozen-thawed cells were recorded. Fig 1 shows that, after freeze-thawing, there was a moderate reduction in the number of PFC/5 10^8 cells The mean survival after freezing was 87 per cent (range 82–95 per cent) of fresh preparations. The difference between fresh and frozen thawed cells was significant ($p<0.05$) in 3 out of 6 experiments. The haemolytic plaques showed the same variations in diameter in both preparations.

Reconstitution of irradiated rats with frozen thawed lymphoid cells Previous studies have shown that injection of both marrow-derived and thymus-derived lymphocytes from normal donors will restore an antibody

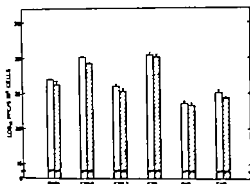


Fig. 1 Effect of freeze-thawing with 10 per cent DMSO on plaque formation by rat spleen cells. Mean alue and 1 standard deviation (SD) of four dishes seeded with 5.10^6 fresh (□) or frozen-thawed (□) cells from the same suspension.

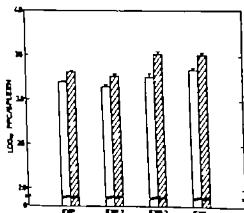


Fig 2 Primary splenic plaque-forming cell response in irradiated rats (800 rad total body X irradiation) restored by 5.10^7 fresh (□) or frozen-thawed (□) mixed spleen and lymph node cells. Mean alue and 1 SD of four dishes seeded with spleen cells pooled from three rats in each group.

response to SRBC in heavily irradiated rats (15 18) The following experiments were designed to study the restoring effect of frozen-thawed lymphocytes

a) *PFC* Rats in groups of three received 800 rad and were subsequently injected i.v with $5\ 10^7$ fresh or frozen-thawed mixed spleen and lymph node cells. Control rats received no cells. One week later they were immunized i.p. with 1 ml 50 per cent SRBC. Preliminary experiments showed that this

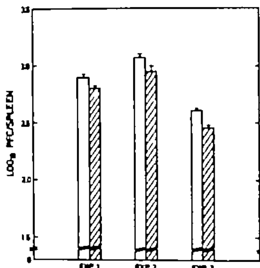


Fig 3 Primary splenic plaque-forming cell response in irradiated rats (800 rad total body X irradiation) restored by 3 10 fresh (□) or frozen-thawed (□) lymph node cells. Mean value and 1 SD of four dishes seeded with spleen cells pooled from three rats in each group.

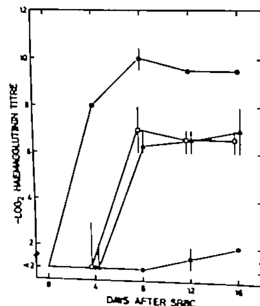


Fig. 4 Mean and range of haemagglutinin response to an intravenous injection of 1 ml 1 per cent SRBC in three rats given 800 rad of whole body X-irradiation and restored with 5.10 fresh (□——□) or frozen-thawed (△——△) mixed spleen and lymph node cells. ○——○ responses of tw normal rats, and ●——● responses of two irradiated rats who were not restored.

delay was needed for the spleens to acquire a measurable number of PFC precursors. Pooled spleen cell suspensions from each group were prepared 4 days later Fig 2 compares the number of PFC in the spleens of rats receiving the frozen thawed suspensions with those restored with fresh cells. The number of PFC/spleen in the control group was always far below 50 and is not shown. It can be seen that the number of PFC/spleen (mean 149 per cent, range 129–188 per cent of fresh) to develop in recipients of frozen thawed cells was higher than that to develop in those receiving corresponding fresh preparations. The difference was significant at a level of $p<0.005$ in all experiments.

Suppressor cells present in spleens, but not in lymph nodes, have been shown to interfere with the response to antigens (8 20) The experiment was therefore repeated using lymph node cells only Fig 3 shows that reconstitution with 3 10^7 frozen thawed lymph node cells gave a number of PFC/spleen (mean 76 per cent, range 71–82 per cent) lower than that obtained by the same number of fresh cells. In these experiments the differences were significant at a level of $p<0.025$

b) *Haemagglutinin titre* 5 10^7 fresh or frozen thawed mixed spleen or lymph node cells and 1 ml 1 per cent SRBC were injected i v into groups of three rats who had received 800 rad on the same day Control animals, 2 in each group were either not irradiated or irradiated without receiving restoring cells. A typical experiment is shown in Fig 4 normal rats rapidly produced a high titre of agglutinating antibody Responses of irradiated restored rats were delayed and did not reach the same high level. There was never any significant difference between recipients of fresh and frozen thawed cells. Irradiated rats receiving no lymphoid cells produced only minimal titres after two weeks

DISCUSSION

In rat SRBC is a thymus dependent antigen (16 18) and T lymphocytes (and probably macrophages) are necessary to obtain an antibody production of the B lymphocytes. The assay of direct PFC is an estimation of the subpopulation of IgM producing B lymphocytes only Previous studies in mice have shown that cryoprotected spleen cells immunized with SRBC can produce antibodies against SRBC after freezing and thawing (1 3, 19)

The present study in rats shows a mean survival of PFC of approximately 85–90 per cent. These results are in accordance with those in mice reported by *Wortis et al.* (19), but far above those reported by *Damjanovic et al* (3) who obtained a survival of only 10–15 per cent after a similar freezing procedure It may be argued that the plaques detected after freezing do not prove that the cells are alive but could depend on leakage of previously produced IgM from dead or damaged cells However production of haemolytic plaques has been shown to be an energy requiring process (3 6) and it is hampered by inhibitors of protein synthesis (3 13) It can therefore be concluded that the plaques are produced by living cells.

Radioresistant B lymphocytes have been demonstrated in rat by *Parish & Hayward* (17) This phenomenon was most pronounced in the secondary response if a proportionally low number of B lymphocytes were used for restoration of the immune response in irradiated animals. It may therefore safely be assumed that in the present experiment using a relatively high number of B lymphocytes in the restoration of a primary response to SRBC in irradiated rats, the antibody produced originates from the progeny of the cells injected and not from the host (5 12, 17)

The higher number of PFC in spleens of recipients of frozen thawed mixed spleen and lymph node cells must be explained Theoretically this would be the result if more T than B lymphocytes were destroyed during freeze-thawing and the T cells left could cooperate with several B cells (9) According to findings in previous experiments, there seems not to be any measurable selective killing of either T or B lymphocytes (10) Thus,

on the assumption that T and B lymphocytes are equally susceptible to freezing damage and are recovered in the same proportion as before freezing, it is reasonable to believe that both groups of rats were injected with T and B lymphocytes in a ratio of about 1 1

T cell dependent suppression of both cell mediated and humoral immune responses has now been demonstrated by several investigators in a variety of different systems (reviewed by *Gershon* 1974) It is shown that such a suppressor cell, probably a spleen-seeking, radiosensitive, thymus-derived lymphocyte is participating in the immune response in mice and rats (2, 4 7 20) In rats, this suppressor phenomenon is most pronounced in younger animals such as those used in the present experiment (7) the finding can well be explained on the basis of inactivation during freeze-thawing of a suppressor activity in the spleen cell suspension. Thus, this liberation from a restrictive control in the immune response to SRBC could more than counterbalance the damage to the PFC precursors in the freeze thaw process. This explanation will be in accordance with previous results obtained by PHA-stimulated frozen-thawed spleen cells (10) Indirect support is provided by the finding that reconstitution by way of frozen-thawed lymph node cells alone gave a number of PFC/spleen lower than that given by corresponding fresh cells. Lymph node cell suspensions are believed to contain significantly smaller amounts of suppressor cells (10, 20)

Macrophages are probably also involved and necessary for the PFC response. It has not yet been proved whether these cells survive the present freeze-thaw process, but as they are radio-resistant they will remain in sufficient numbers in the recipient after irradiation (4 5)

The haemagglutinating antibody titre is a semi-quantitative assay and therefore it can only be concluded that there was no great difference between fresh and frozen-thawed cells in the ability to restore the haemagglutinating antibody response. Certainly higher titres could have been obtained in the restored rats if the dose of cells used for injection had been higher 5 10 cells are probably only approximately 5 per cent of the total number of lymphocytes in a normal rat (5)

REFERENCES

1 *Albright, J F Makinodan T & Mazur P* Preservation of antibody-producing cells at low temperatures a method of storage that allow complete recovery of activity Proc. Soc. exp. biol. med. *114* 489-493 1963.

2. *Bell, E. B. & Shand F L.* Changes in lymphocyte recirculation and liberation of the adoptive memory response from cellular regulation in irradiated recipients. Eur J Immunol. *5* 1 7 1975.

3. *Damjanovic V., Edwards D C & Thomas D* Recovery of haemolytic plaque-forming cells after freeze-drying Nature *253* 116–119 1975.

4 *Ellis S T., Gowans, J L. & Howard J C* Cellular events during the formation of antibody Cold. Spr Harb. Symp. quant. Biol. *32* 395–406 1967

5. *Ellis, S T., Gowans, J L. & Howard J C* The origin of antibody-forming cells from lymphocytes Antibiot. et Chemoter (Basel) *15* 40–55 1969

6 *Fitch, F W., Rosenman J M Rowley D & Bernbaum M C* Cell respiration as a requirement for antibody release in vitro. Nature *218* 972–973 1968.

7 *Folch, H & Waksman B* The splenic suppressor cell. I. Activity of thymus-dependent adherent cells changes with age and stress. J Immunol *113* 127 139 1974

8 *Gershon R. K.* T cell control of antibody production Contemp. Top. Immunobiol *3* 1–40, 1974.

9 *Haber Katz, E. & Wilson, D B.* Collaboration of allogeneic T and B lymphocytes in the primary antibody response to sheep erythrocytes in vitro. J exp. Med. *142* 928–935, 1975

10 *Hem K.* Freezing of rat lymphocytes. I The effects of dimethyl sulphoxide and freezing on the phytohaemagglutinin and pokeweed mitogen responding lymphocyte subpopulations. Cryobiology *13* 134–141 1976

11 *Hem, K. & Sokolowski, J* Freezing of rat lymphocytes II Distribution and circulation of frozen-thawed Cr-labelled cells. Cryobiology in press 1976.

12. *Howard J C & Gowans J L.* The role of lymphocytes in antibody formation. III. The origin from small lymphocytes of cells forming

direct and indirect haemolytic placques to sheep erythrocytes in the rat. Proc. R. Soc. Lond. B. *182* 193–209 1972

13 *Ingram J S & Bussard A* Application for a localized hemolysin reaction for specific detection of individual antibody forming cells. J exp Med. *119* 667–683 1964

14 *Jerne N U Nordin A A & Henry C* The agar plaque technique for recognizing antibody producing cells. In *Amos B. & Koprowski H* (Eds.) Cell-bound antibodies. Wistar Institute Press, Philadelphia, 1963 pp 109–122.

15 *Johnston J & Wilson D B* Origin of immunoreactive lymphocytes in rats. Cell. Immunol *1* 430–444 1970

16 *Knight S C Newey B & Ling, N R* Thymus dependence in the rat of lymphocytes responding to stimulation with specific antigen, allogenic lymphocytes and non-specific mitogens. Cytobios 7 35–49 1973

17 *Parish C R. & Hayward J A* The lymphocyte surface. III Function of Fc receptor C_3 receptor and surface IgG bearing lymphocytes Identification of a radioresistant B cell. Proc. R. Soc. Lond B. *187* 379–395 1974

18 *Scott D W & Howard J C* Collaboration between thymus-derived and marrow-derived thoracic duct lymphocytes in the hemolysin response of the rat. Cell Immunol. *3* 430–441 1972

19 *Wortis H H Taylor R. B & Dresser D W* Antibody production studied by means of the localized hemolysis in gel (LHG) assay Immunology *14* 69–79 1968

20 *Wu C Y & Lance E M:* Immunoregulation by spleen seeking lymphocytes. II Role in the response to sheep erythrocytes. Cell. Immunol *13* 1–11 1974

cta path. microbiol. scand. Sect. C, 84: 495–500, 1976

INHIBITION OF THE MITOGENICITY OF THE CARRIER MOLECULE RESULTS IN LOSS OF IMMUNOGENICITY OF A HAPTEN-LPS CONJUGATE

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Smith, E. & Hammarström, L. Inhibition of the mitogenicity of the carrier molecule results in loss of immunogenicity of a hapten-LPS conjugate. Acta path. microbiol. scand. Sect. C, 84: 495–500, 1976.

Colistin methanesulfonate, a basic polypeptide similar to polymyxin E, has been shown to suppress the mitogenicity of lipopolysaccharide (LPS) from *E. coli*. It also inhibits the immunogenicity of a hapten-LPS conjugate. The inhibition was neither due to interference with the expression of hapten determinants, nor was it due to crossreactivity between the hapten and colistin methanesulfonate. As mitogenicity and immunogenicity was similarly affected, we conclude that activation of bursa-derived lymphocytes, in specific thymus-independent immune responses, does not take place in the absence of a mitogenic (non-Ig mediated) signal, thus supporting the hypothesis of the "one nonspecific signal" for B cell triggering.

Key words: Colistin methanesulfonate; hapten-lipopolysaccharide conjugates; lipopolysaccharide mitogenic inhibitors; polymyxins.

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Antigens, that directly activate resting bursa-derived lymphocytes (B cells) to proliferation and antibody synthesis, without helper effects from thymus-derived lymphocytes (T cells), are called thymus-independent (TI) antigens. TI antigens like lipopolysaccharide (LPS) are also called B cell mitogens or polyclonal B cell activators (3). Thymus-independent induction of antibody production is the simplest model for the study of B cell triggering and different models have been proposed to explain this system.

Most TI antigens have repeating antigenic determinants and thus it has been suggested that the interaction of these polymeric antigens with these specific immunoglobulin-receptors (Ig-receptors) would be sufficient to induce proliferation and antibody synthesis (8) in specific B cells. A competing hypothesis, however, claims that there is only a nonspecific mitogenic signal that triggers cells and that the Ig-receptors only passively focus non-specific triggering signals onto the cell membrane (3). The latter possibility is supported by the finding that all TI-antigens are mitogenic themselves in high concentrations (6).

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with NNP was determined. As can be seen in Table 1 the induction of antibody production was inhibited to a similar extent as the proliferative response. As CM at a concentration above 200 μg/ml proved to be toxic under our experimental conditions, and subsequently could not be used the inhibition was not complete and high doses of LPS could still amount a good polyclonal antibody response. However CM in a concentration of ≤ 100 μg/ml did not affect all viability and e.g. phytohaemagglutinin (PHA) stimulated cells mounted a normal response after the addition of CM (Table 2)

TABLE 1 *Inhibition of LPS I duc d Poly lo al Anti-NNP PFC by Colistin M thane ulfonat PFC/cult Day 2*

LPS μg/ml	CM/ml		
	0 μg	10 μg	100 μg
1000	2760	4020	4480
100	4640	3760	1740
10	3720	1760	580
1	3640	1180	480
10	2800	540	460
10	2420	640	460
10	2220	720	480
0	820	520	540

LPS was reacted with colistin methanesulfonate and the mixture was added to cultures which were set up with 10^7 spleen cells/ml/culture medium containing 5 per cent fetal calf serum and 5×10^{-5} M 2-mercaptoethanol

TABLE 2 *Eff ct of CM Addition on H-thymidine Incorporation in PHA-stimulated Mouse Spleen Cells*

PHA μg/ml	CM/ml		
	0 μg	10 μg	100 μg
10	112320	105930	111320
1	153270	161350	150690
0	7750	6950	7420

PHA was reacted with colistin methanesulfonate and the mixture was added to cultures which were set up with 10^6 spleen cells/ml/culture in medium containing 5 per cent fetal calf serum and 5×10 M 2-mercaptoethanol

Results are given in CPM

CM Inhibits Immunogenicity of Hapten-LPS Conjugates

Since mitogenicity of LPS was decreased after the addition of CM we also wanted to use hapten-LPS conjugates and study their interaction with CM The effect on these conjugates would give evidence for how specific immune cells are activated. If PB would have no influence, this would implicate that the mitogenic properties of the LPS molecule are of no importance for specific thymus-independent immune responses. If on the other hand, the addition of PB would lead to decreased mitogenicity of these conjugates, this would support the theory that claims, that the activation of B cells is due to a non-specific signal from the carrier molecule, this molecule itself merely passively being focused to the target cell via Ig-receptors for the haptenic determinants. As can be seen from Fig 2, the addition of CM to hapten-LPS stimulated spleen cell cultures leads to a shift in the PFC dose response curve. The most PFC stimulatory NNP LPS concentration is higher after CM addition. This establishes that when the mitogenic part of a hapten-LPS molecule is affected, it leads to a changed immunogenicity of this complex. The increased PFC values that are seen in spite of the shift in the dose response curve are probably due to a decreased LPS toxicity

To demonstrate that CM and antigen does not crossreact, that is, inhibition of LPS is nonspecific, CM was used in the plaque assay system The safest way to assess possible crossreactivity namely appeared to be, to test the crossreactivity of the PFC we were obtaining in the response of the antigen, and on the same target cells where the above results were obtained. In this way not only the specificity but also the avidity of the activated cells could be controlled and the cells which were tested, were actually the cells which could be inhibited in the presence of CM. CM was incorporated into the agar used for the assay of PFC with cells that had been activated by NNP LPS *in vitro* As can be seen in Table 3 no inhibition was detected.

One possible explanation for the inhibitory

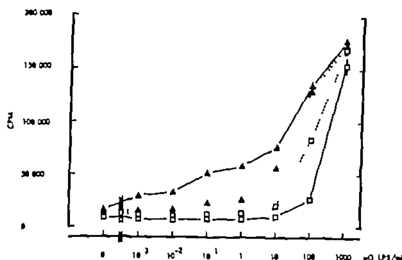


Fig 1 Inhibition of proliferative response to LPS on day 2 by addition of 0 (Δ—Δ) 1 (Δ Δ) 10 (□ □) or 100 μg/ml (□—□) of colistin methanesulfonate. Cultures were set up in microcultures with 500 000 spleen cells/well in medium containing 5 per cent fetal calf serum and 5 × 10⁻⁵ M 2 mercaptoethanol. The values given are means of 3 cultures, vertical bars representing S.E.

In order to distinguish between these alternatives, we have used colistin methanesulfonate (CM) which is a basic polypeptide produced by *Bacillus colistinus*. The structure resembles polymyxin E and it can thus be regarded as belonging to the polymyxin group of antibiotics. Other polymyxins have previously been shown to prevent the toxicity of LPS (12) neutralize the Schwartzman reaction (13) and inactivate the mitogenic properties of LPS (2 9 14)

MATERIALS AND METHODS

Mice B10.5M (H 2ᵇ) mice of both sexes, 4–8 weeks of age from our own colony were used in all the experiments.

Culture conditions The method for the study of polyclonal proliferative responses to LPS and LPS-PB conjugates as well as for the induction of specific thymus-independent anti NNP antibody responses by NNP LPS has been described earlier (7 4) Cultures were supplemented with 5 per cent fetal calf serum (FCS) and 2 mercaptoethanol (2 ME) at a concentration of 5×10^{-5} M

LPS LPS from *E. coli* 055 B5 obtained by phenolwater extraction (17) was used throughout these experiments. The hapten NNP was conjugated to LPS as previously described (4) and the biological characterization of the conjugate used in the present experiments has been presented before (5)

Colistin methanesulfonate CM (batch 176/2665) was a gift from AB H Lundbeck Co, Sweden.

Phytohaemagglutinin PHA was obtained from Wellcome Research Laboratories, Beckenham Kent.

Detection of antibody-producing cells The number of antibody-producing cells (PFC) to the hapten NNP (4-hydroxy 3,5-dinitro-phenacetyl) was determined by the use of a modification of the haemolytic plaque assay in agar (1) for detecting low affinity (polyclonal) or high-affinity (specific) anti hapten PFC.

Inhibition of rosette forming cells NNP LPS was mixed with different amounts of PB and allowed to interact for 15 minutes at room temperature. Anti NNP spleen cells from hyperimmunized mice were admixed and left for additional 15 minutes at room temperature Without subsequent washing NNP-coated SRBC were added and the suspensions centrifuged for 10 minutes at 4 C to allow formation of RFC. Details of this method have been published elsewhere (11)

RESULTS

CM Inhibits Mitogenicity of LPS

In a first set of experiments we determined the ability of CM to inhibit LPS induced B cell proliferative responses. Microcultures were set up with various amounts of LPS. CM was added and the mixture was allowed to react for 15 minutes after which the spleen cells were added. The results of such an experiment are given in Fig 1 As can be seen increasing doses of CM cause a decrease in mitogenicity Treatment with CM did not completely abolish mitogenicity but much higher LPS doses were required to achieve the same level of responses. The effect of CM on polyclonal antibody synthesis was investigated in a similar way Petri dishes containing LPS was reacted with CM Spleen cells were added and the plaque forming cell (PFC) response to SRBC heavily coupled

with NNP was determined. As can be seen in Table 1 the induction of antibody produc tion was inhibited to a similar extent as the proliferative response. As CM at a concentration above 200 µg/ml proved to be toxic under our experimental conditions, and subsequently could not be used, the inhibition was not complete and high doses of LPS could still amount a good polyclonal antibody response. However CM in a concentration of ≤100 µg/ml did not affect all viability and e.g. phytohaemagglutinin (PHA) stimulated cells mounted a normal response after the addition of CM (Table 2).

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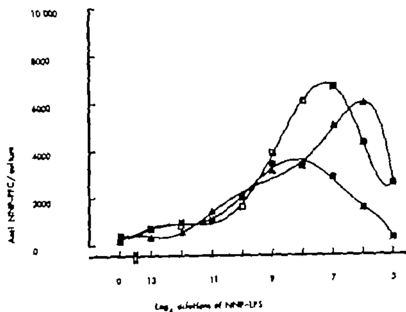


Fig 2 Induction of specific anti NNP plaque-forming cells with NNP LPS on day 3 with addition of 0 (■) 10 (□) or 100 µg/ml (△) of colistin methanesulfonate. Cultures were set up in plastic petri dishes with 10^7 spleen cells/culture in medium containing 5 per cent fetal calf serum and 5×10^{-5} M mercaptoethanol.

TABLE 3 *Effect of Incorporation of Free Colistin Methanesulfonate in the Anti-NNP PFC Assay*

CM µg/ml	PFC/culture induced by 7 log dilution of NNP LPS	PFC/culture induced by 9 log dilution of NNP LPS	Control PFC/culture
0	1720	600	90
10^{-2}	1970	540	90
10^{-1}	1780	520	70
1	1460	560	90
10	1860	730	70

Cells from the same cultures shown in Fig 2 were also tested in the same plaque assay with incorporation of free colistin methanesulfonate in the agar at the time of plating. Cultures were set up in plastic petri dishes with 10^7 spleen cells/culture in medium containing 5 per cent fetal calf serum and 5×10^{-5} M 2-mercaptoethanol. Cells were stimulated with NNP LPS which was reacted with colistin methanesulfonate and assayed on day 3

effect of CM on NNP LPS conjugates is that the NNP determinants could be blocked. The binding of the conjugate to NNP specific lymphocytes would, if this was the case be affected To demonstrate that this is not the cause of the inhibition the rosette inhibition assay was used. Since the RFC inhibition assay depends on the valency of the conjugates (10) as well as on the total amount of hapten in the reaction this test is well suited for detecting the pattern of determinant presentation to the specific cells. Lymphocytes from mice, which had been immunized several times with NNP conjugates, were used as RFC CM was reacted with the NNP LPS conjugate and thereafter the mixtures were allowed to react with a spleen cell suspension containing a high number of high-avidity hapten binding cells. It was clearly shown that the inhibitory effect of NNP LPS, which prevents the NNP specific lymphocytes from binding to the NNP coupled target cells, was not affected by the addition of CM (Table 4)

TABLE 4 *Rosette Inhibition of Hyperimmune Anti-NNP Spleen Cells with NNP LPS Conjugates Reacted with Colistin Methanesulfonate Results Given as Per Cent RFC*

Log_4 dilution of NNP LPS	CM µg/ml 0 µg	10 µg	100 µg
5	3 5	4.5	4 0
7	4 0	4 6	4 7
9	7.5	6.8	7 0
11	7 0	7 7	6 8
13	7 2	7 0	6 0
0	7 0	6.5	8 0

NNP LPS was preincubated with colistin methanesulfonate. 25×10^6 NNP hyperimmune spleen cells were added and the mixture was incubated for 30 minutes at room temperature Thereafter NNP SRBC were added and the cells were centrifuged at 4 C for 10 minutes resuspended and assayed for per cent rosette forming cells

This demonstrates that the haptenic groups are intact and not blocked by CM.

DISCUSSION

The present results show that the mitogenicity of LPS, as well as the immunogenicity of a hapten-LPS conjugate is abolished by colistin methanesulfonate. This is in accordance with our previous results (16) achieved in a different culture system, using polymyxin B, a basic polypeptide of the same family as CM. Earlier results dealing with the toxicity of different polymyxins have in fact suggested that CM and polymyxin E are the same. Since CM is far less toxic (12) than PB, it proved to be a useful tool in our line of research, surveying the mechanism of B cell triggering. The inhibition of the immunogenicity of NNP LPS could be overcome by higher concentrations of the conjugate, and the high-dose unresponsiveness was also obtained at a higher dose in the presence of the inhibitor. This was not due to cross-reactivity between CM and the hapten, nor was it due to blocking of the haptenic determinants. Thus we conclude that B cell triggering in specific thymus-independent immune responses is dependent upon the intrinsic mitogenicity of the carrier molecule and not upon the pattern of antigen presentation. Although the role of an Ig-mediated signal is not completely ruled out, these experiments would then tend to support the "one non-specific signal" hypothesis (3) that has been postulated for B cell triggering.

These findings are in complete contrast to previous results (9) using TNP LPS and PB where the immunogenicity of the conjugate was unaffected although the mitogenicity of LPS was abolished. In our previous paper (16) using NNP LPS and PB, we reported that the immunogenicity and mitogenicity decreased in parallel. However since these results were obtained in a completely different culture system, the reason for these discrepancies remained unsolved. We have now confirmed our own findings using a culture system which includes fetal calf serum and 2 ME. The shift in the dose response curve is, however smaller than under serum free conditions which could be due to the extra amount of LPS added to the cultures via contaminated FCS (15) and further work, in collaboration with *D. Jacobs* and *D. Morrison* is presently being carried out to determine the role of FCS in specific thymus-independent B cell triggering.

The technical assistance of Ms. *Ylva Atellan* is gratefully acknowledged. This work was supported by *Olle and Elof Ericsson's Foundation* the *Eva and Oscar Ahréns Foundation* and the *Anders Otto Swärd Foundation*.

REFERENCES

1. *Bullock, W. W. & Möller, E.*: Spontaneous B cell activation due to a loss of a normal serum suppressor. Eur. J. Immunol. *2* 514–517, 1972.
2. *Bulls, T. Hammarström, L., Smith, E. & Möller, G.*: Inhibition of polyclonal B cell activators. Infect. and Immun. (In press)
3. *Coutinho, A.*: The theory of the "one non-specific signal" model for B cell activation. Transpl. Rev. *23* 49–65, 1975
4. *Coutinho, A. Gronowicz, E. Bullock, W. W. & Möller, G.*: Mechanism of thymus-independent immunocyte triggering. Mitogenic activation of B cells results in specific immune responses. J. Exp. Med. *139* 74–92, 1974
5. *Coutinho, A. Gronowicz, E. & Möller, G.*: The role of Ig receptors in antigen induced activation of B lymphocytes. Progr. Immunol. 2 167–175, 1974
6. *Coutinho, A. & Möller, G.*: B cell mitogenic properties of thymus-independent antigens. Nature New Biol. *245* 12–14, 1973
7. *Coutinho, A. Möller, G. & Richter, W.*: Molecular basis of B cell activation. I. Mitogenicity of native and substituted dextrans. Scand. J. Immunol. *3* 321–328, 1974
8. *Feldmann, M. & Basten, A.*: The relationship between antigenic structure and the requirement for thymus-derived cells in the immune response. J. Exp. Med. *134* 103–119, 1971
9. *Jacobs, D. & Morrison, D.*: Dissociation between mitogenicity and immunogenicity of TNP Lipopolysaccharide, T-independent antigen. J. Exp. Med. *141* 1453–1458, 1975
10. *Möller, E.*: Specificity of hapten-reactive T and B mouse lymphocytes. Scand. J. Immunol. *3* 339–355, 1974

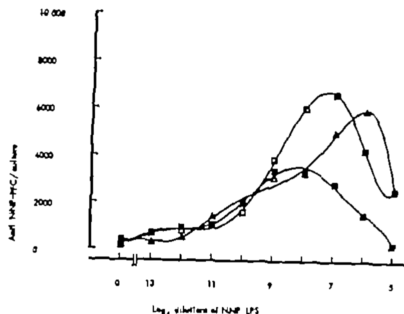


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REFERENCES

1. *Bullock W. W. & Möller E.*. Spontaneous B cell activation due to loss of a normal serum suppressor. Eur. J. Immunol. 2: 514–517, 1972.
2. *Butler T., Hammarström L., Smith E. & Möller G.*. Inhibition of polyclonal B cell activation. Infect. and Immun. (In press)
3. *Coutinho A.* The theory of the "one non-specific signal" model for B cell activation. Transpl. Rev. 23: 49–65, 1975.
4. *Coutinho A., Gronowicz, E., Bullock W. W. & Möller G.* Mechanism of thymus-independent immunocyte triggering. Mitogenic activation of B cells results in specific immune responses. J. Exp. Med. 139: 74–92, 1974.
5. *Coutinho A. Gronowicz E. & Möller G.* The role of Ig receptors in antigen induced activation of B lymphocytes. Progr. Immunol. 2: 167–175, 1974.
6. *Coutinho A. & Möller G.* B cell mitogenic properties of thymus-independent antigens. Nature New Biol. 245: 12–14, 1973.
7. *Coutinho A. Möller G. & Richter W.* Molecular basis of B cell activation. I. Mitogenicity of native and substituted dextrans. Scand. J. Immunol. 3: 321–328, 1974.
8. *Feldmann M. & Basten, A.* The relationship between antigenic structure and the requirement for thymus-derived cells in the immune response. J. Exp. Med. 134: 103–119, 1971.
9. *Jacobs D. & Morrison D.*. Dissociation between mitogenicity and immunogenicity of TNP-Lipopolysaccharide, a T-independent antigen. J. Exp. Med. 141: 1453–1458, 1975.
10. *Möller E.* Specificity of hapten-reactive T and B mouse lymphocytes. Scand. J. Immunol. 3: 339–355, 1974.

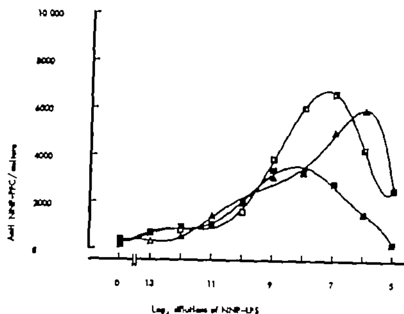


Fig 2 Induction of specific anti-NNP plaque-forming cells with NNP LPS on day 3 with addition of 0 (■) 10 (□) or 100 μg/ml (△) of colistin methanesulfonate. Cultures were set up in plastic petri dishes with 10^7 spleen cells/culture in medium containing 5 per cent fetal calf serum and 5×10^{-5} M 2-mercaptoethanol.

TABLE 3 *Effect of Incorporation of Free Colistin Methanesulfonate in the Anti NNP PFC Assay*

CM μg/ml	PFC/culture induced by 7 $\log_4$ dilution of NNP LPS	PFC/culture induced by 9 $\log_4$ dilution of NNP LPS	Control PFC/culture
0	1720	600	90
10^{-2}	1970	540	90
10^{-1}	1780	520	0
1	1460	560	90
10	1860	730	70

Cells from the same cultures shown in Fig 2 were also tested in the same plaque assay with incorporation of free colistin methanesulfonate in the agar at the time of plating. Cultures were set up in plastic petri dishes with 10^7 spleen cells/culture in medium containing 5 per cent fetal calf serum and 5×10^{-5} M 2-mercaptoethanol. Cells were stimulated with NNP LPS which was reacted with colistin methanesulfonate and assayed on day 3

effect of CM on NNP LPS conjugates is that the NNP determinants could be blocked The binding of the conjugate to NNP specific lymphocytes would if this was the case be affected To demonstrate that this is not the cause of the inhibition the rosette inhibition assay was used. Since the RFC inhibition depends on the valency of the conjugates (10) as well as on the total amount of hapten in the reaction this test is well suited for detecting the pattern of determinant presentation to the specific cells Lymphocytes from mice which had been immunized several times with NNP conjugates were used as RFC CM was reacted with the NNP LPS conjugate and thereafter the mixtures were allowed to react with a spleen cell suspension containing a high number of high-avidity hapten binding cells. It was clearly shown that the inhibitory effect of NNP LPS which prevents the NNP specific lymphocytes from binding to the NNP coupled target cells, was not affected by the addition of CM (Table 4)

TABLE 4 *Rosette Inhibition of Hyperimmune Anti NNP Spleen Cells with NNP LPS Conjugates Reacted with Colistin Methanesulfonate Results Given as Per Cent RFC*

$\log_4$ dilution of NNP LPS	CM μg/ml 0 μg	10 μg	100 μg
5	3.5	4.5	4.0
7	4.0	4.6	4.7
9	7.5	6.8	7.0
11	7.0	7.7	6.8
13	7.2	7.0	6.0
0	7.0	6.5	8.0

NNP LPS was preincubated with colistin methane sulfonate. 25×10^6 NNP hyperimmune spleen cells were added and the mixture was incubated for 30 minutes at room temperature. Thereafter NNP SRBC were added and the cells were centrifuged at 4 C for 10 minutes resuspended and assayed for per cent rosette forming cells

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IMMUNOLOGICAL *IN VITRO* PARAMETERS IN PATIENTS WITH MULTIPLE SCLEROSIS AND IN NORMAL INDIVIDUALS

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The Tissue Typing Laboratory, University Hospital, Copenhagen, and
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Platz, P., Fog, T., Morling, N., Svejgaard, A., Sonderstrup, G., Ryder, L. P., Thomsen, M. & Jersild, C. Immunological *in vitro* parameters in patients with multiple sclerosis and in normal individuals. Acta path. microbiol. scand. Sect. C, 84: 501-510, 1976.

The general immunological capacity of 40 patients with multiple sclerosis has been evaluated with lymphocyte transformation test including both mitogens (PHA and PWM) and antigens (PPD, *Candida albicans, St. ph. aureus* and *E. coli*). Determination of T and B cells was performed by E-, EAC-rosetting and immunofluorescence for surface immunoglobulins. Compared with the results obtained in 42 normal individuals only minor differences were found.

Key words: Multiple sclerosis; lymphocyte transformation test; T and B cells.

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The aetiology and pathogenesis of multiple sclerosis (MS) is still unknown despite intensive research. However, within the latest years, it has become clear that genetic as well as environmental and immunological factors are involved in the occurrence and course of this disease.

It has been shown by several groups (for review see *Jersild et al.* (18)) that genetic factors (HLA-A3, B7 and Dw2) of the major histocompatibility system (HLA) are found at increased frequencies in patients with MS. The mechanism by which these HLA markers or closely linked determinants confer susceptibility to MS is unknown, but it was found that the course of MS is influenced by the presence of the Dw2 determinant, since rapid progression occurred predominantly in patients carrying this determinant (19, 32).

Several environmental factors have been suspected in MS: nutritional (4, 28), toxic (8) and infectious agents, especially viruses. Evidence for the latter is still mostly circumstantial: increased frequency of high titre antibodies to measles and para-influenza viruses in MS patients seems well established (6, 33). *Norrby & Vandvik* (29) found in some MS cases that the oligoclonal IgG in cerebrospinal fluid contained antibody activity against measles virus, but this could not be found in other cases. More recently *Koldovsky et al.* (22) have been able to confirm the findings obtained by *Carp et al.* (9) that brains from MS cases contain materials which in serial passages in the mouse showed evi-

11 *Möller E Bullock W W & Mäkelä O* Affinity of T and B lymphocyte receptors for hapten determinants. Eur J Immunol. *3* 172–179 1973
12 *Nord N M & Hoeprich P D* Polymyxin and Colistin. A critical comparison. New Engl. J Med. *270* 1030–1035 1964
13 *Rifkind D* Prevention by polymyxin B of endotoxin toxicity lethality in mice. J Bacteriol. *93* 1463–1464 1967
14 *Rifkind D & Hill R B* Neutralization of the Schwarzman reactions by polymyxin B. J Immunol. *99* 564–569 1967
15 *Shugi S M & Mishell R I.* Sera and the in vitro induction of immune responses. I Bacterial contamination and the generation of good fetal bovine sera. J Immunol *115* 741–744 1975
16 *Smith E. Hammarström L. & Coutinho A* Association between mitogenicity and immunogenicity of NNP lipopolysaccharide, a T-independent antigen J Exp Med. *143* 1521–1527 1976.
17 *Westphal O Lüderitz O & Bister F.* Über die extraktion von Bakterium mit Phenolwasser 2 Z. Naturforsch. Teil B. *7b* 148–155 1952

stimulation) or 170 hours (PWM 120 h and antigen stimulation). To each tube 0.05 μCi of ^{14}C-thymidine (specific activity 0.25 mCi/mg NEN Chemicals) was added 24 hours before termination of the cultures. The cells were harvested on glass fibre filters (SKATRON, Oslo) using Skatron semi-automatic harvesting machine and washed with distilled water. Liquid scintillation counting of the filters was done in 5 ml of Instagel (Packard). The stimulation was expressed as counts per minute (cpm) in stimulated cultures minus cpm in unstimulated cultures (increment cpm). The median cpm of the triplicates was used for calculations.

Mitogens and Antigens

T each culture tube (500 μl) 50 μl of mitogen or antigen dilution in medium was added. The following dilutions and concentrations are the final ones in the culture tubes. Phytohaemagglutinin (PHA-P Difco). A stock solution of 1 ampoule plus 5 ml distilled water was diluted 1:60 to a final concentration of 400 μg/ml, 1:600 (40 μg/ml), 1:2400 (10 μg/ml) and 1:6000 (4 μg/ml). Pokeweed mitogen (PWM, Gibco). A stock solution prepared like PHA was diluted 1:500.

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The day-to-day variation in the lymphocyte transformation was investigated in the following way:

(i) Two normal individuals were examined, using PHA 40 μg/ml in the same set up at the same day on 59 different occasions over the 18 months period.

(ii) The level of responsiveness to mitogens and antigens was followed in two other normal individuals both tested 49 times during the 18 months period.

Rosett Forming Cells

8 ml of the blood medium mixture was incubated with 0.8 g carbonyl iron and 3–6 glass beads at 37 C for 20 min in plastic tube which was slowly rotated. The phagocytosing cells were sedimented with powerful magnet and the remaining cells were isolated on Lymphoprep. The lymphocytes were washed three times in Hanks solution and resuspended in Hanks balanced salt solution (HBSS) at concentration of 3×10^6 cells per ml (normally containing less than 1 per cent phagocytosing cells).

E-rosettes

The method described by *Bentwich et al.* (3) was used with the following modifications. 100 μl of purified lymphocyte suspension was incubated with 100 μl of an 0.5 per cent suspension of sheep red blood cells (SRBC) and 20 μl AB serum (absorbed with SRBC) for 30 minutes at 37 C in a Falcon plastic tube.

The mixture was centrifuged at 40 *G* for 5 minutes and incubated overnight at 4 C, the samples were done in duplicate, 100 cells were counted and the mean of the two counts was determined.

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A modification of the method described by *Mendes et al.* (27) was used with the following modifications. Antibody-complement coated erythrocytes were prepared in the following way: *Human A erythrocytes were washed three times in* HBSS and resuspended to a concentration of 2.5 per cent (v/v). The suspension was incubated at 37 C for 30 min with an equal volume of IgM rabbit-anti-A-antiserum (a gift from Statens Seruminstitut) diluted to an optimal non-agglutinating concentration (1:2500). Two ml of the suspension of antibody coated erythrocytes were incubated with 100 μl mouse serum (complement source) for 30 min at 37 C and finally diluted with 3 ml HBSS.

For the rosette assay *100 μl lymphocyte* suspension was mixed with 100 μl erythrocyte suspension in Falcon tubes and centrifuged for 5 min at 40 *G* whereafter 20 μl 0.1 per cent toluidine blue was added. After 5 minutes the cell pellet was resuspended and counted.

Immunoglobulin Bearing Cells

The blood medium suspension was layered on Lymphoprep and the mononuclear cells were isolated; they were washed three times in HBSS and transferred to tubes at a concentration of 10^6 cells per ml. The cells were incubated with 1 ml of a 1 per cent latex (Difco, particle size 0.8 μm) suspension in HBSS for 30 min at 37 C. The cells were isolated and the following steps carried out at 0° C to avoid capping. Three drops of 1:7 dilution of FITC conjugated polyvalent rabbit-anti-human Ig (Dako, Copenhagen) was added and incubated for 30 min. The non-bound conjugate was removed by washing twice with 5 per cent human albumin in HBSS and the cells were resuspended in one drop of 67 per cent fluorescence free glycerol (Merck). The wet slides were examined at magnification of 675 × in a Leitz Orthoplane fluorescence microscope with incident light (Xe 150) equipped with the following filters: barrier filter Bg38 (4 mm), dicroic filter (K490), secondary

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The increased antibody titres against para myxoviruses and the oligoclonal IgG in Cerebro Spinal Fluid (CSF) from MS patients (24) were some of the first signs to provide evidence of an involvement of the immunological system in MS

The increasing numbers of *in vitro* tests for Cell Mediated Immunity (CMI) have given somewhat conflicting results when applied to the study of MS Results obtained by a direct leucocyte migration test suggest a selective, decreased reactivity against some paramyxoviruses, especially measles (11 16 32 36 37) However these results could not be confirmed by others (2) and studies using the lymphocyte transformation test with measles antigen (12 21) show a normal responsiveness of MS lymphocytes to this virus. Differences in antigen preparations are known to influence test results and can easily explain the discrepancies between these results.

The response to PHA in the lymphocyte transformation test was at first found decreased (17 23) in cultures with autologous serum later MS serum was found to contain factors which reduce the PHA response also in normal individuals (10 14 20) The PHA responsiveness in pooled serum has been found normal by most investigators (5 13 34) but decreased by others.

The lymphocyte subpopulations (T and B lymphocytes) in MS patients have recently been investigated *Lisak and coworkers* (25) found a decrease in relative numbers of T cells (determined by SRBC-rosettes) in MS patients during acute exacerbations but not during stable periods *Oger and coworkers* (31) found a decrease in the number of avid' T rosettes, (>10 SRBC per lymphocyte) but the deviation was not more pronounced in patients with active disease. Both groups found an increase in number of B cells in MS patients. *Nowak & Wajgt* (30) found a normal percentage of T lymphocytes but a reduction of lymphocytes bearing C3 and Fc receptors.

In an attempt to evaluate the general immunological capacity in MS patients we investigated 40 randomly selected MS patients, all in a stable phase of the disease using the lymphocyte transformation test with PHA, pokeweed mitogen (PWM) and various antigens as well as enumeration of T and B cells in the peripheral blood.

MATERIAL AND METHODS

A total of 40 randomly selected patients with MS 23 male and 17 female (mean age 43 years) was investigated. All patients presented a definite diagnosis of MS fulfilling the criteria set up by *Broman et al.* (7) and all were seen by one of us (TF) The patients were either from the MS outpatients clinic at the Municipal Hospital, Copenhagen, or from the MS Rehabilitation Centre in Hasley None of the patients were in an acute phase of disease at the time of investigation.

The control group was composed of blood donors and members of the hospital staff 25 females and 17 males (mean age 30 years and 6 months) The controls were tested over an 18 months period they were randomly selected in order that each month might be represented by at least 2 persons and each person only by one test. Two pairs of individuals were tested repeatedly and simultaneously to investigate the day to-day variation of the transformation test.

Blood Samples

Blood was drawn into an equal volume of RPMI 1640 (Bio-cult) with 15 iu heparin without preservatives (Novo, Denmark) per ml

Lymphocyte Transfo mation

The blood medium mixture was layered on Lymphoprep (Nyegaard, Oslo, Norway) centrifuged at 500 *G* for 20 minutes, the mononuclear cells were isolated from the interphase and washed three times in RPMI 1640 (with 25 mM hepes) supplemented with 5 per cent pooled human serum from non-transfused young male donors. The cultures (all done in triplicates) were set up in tubes (NUNC, No 1072) containing 10^5 cells in 0 5 ml RPMI 1640 supplemented with 15 per cent serum To each ml of medium was added 15 u heparin 500 u penicill n (Leo Denmark) 500 µg streptomycinbase nd 1 ? µmoles glutamine After addition of mitogen and antigens in arious concentrations, the cells were cultured n 5 per cent CO in humid f d a r at 37 C for 96 hours (mitogen

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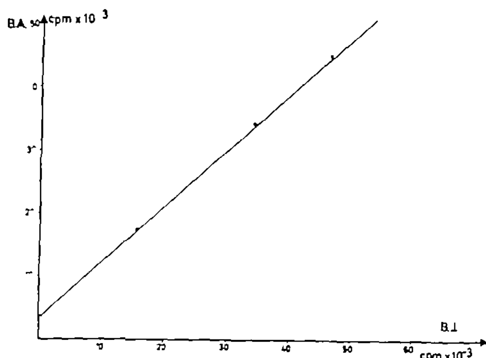


Fig 1 Correlation between the response to PHA 40 µg/ml in two normal individuals, B.A and B J obtained by 39 different tests.

filter K510 Of the non phagocytosing cells 100 were examined and the percentage of cells with surface fluorescence determined

Statistics

All results are given as the median and the range of observations since neither the observed values themselves nor the logarithm of these values fit the normal distribution according to Gauss.

Statistical comparisons were made by the Mann-Whitney rank sum test, and correlations were tested by the non parametric Kendall rank-correlation test (1)

RESULTS

Lymphocyte Transformation

1 Reproducibility Examination of the same two normal individuals was repeated in an attempt to investigate the day to-day variation of the test. Fig. 1 shows the results obtained by tests of these two individuals who were tested 39 times by PHA 40 µg/ml over an 18 months period. The highly significant ($p<10^{-4}$) correlation between these two individuals responsiveness at different times, indicate that the technical performance of the test greatly influences the results.

The variation in individual responsiveness to antigens and mitogens was followed in two other normal persons, who were tested 49 times during the 18 months period. In both individuals, a significant correlation between the responsiveness to mitogens (PHA, PWM) and antigens (PPD candida) was found ($p<0$ 01 and $p = 0$ 03 respectively (Kendall rank-correlation))

2 Mitogens Only minor deviations were found when results from MS patients were compared with those obtained in normal individuals. The dose-dependent responsiveness to different concentrations of PHA (Fig 2) was similar in the two groups although the response to the highest concentration of PHA (PHA 400 µg/ml) was higher in the MS group The responsiveness to PWM was the same in both groups. Table 1 gives the median and range in both groups.

3 Antigens The transformation with PPD *Candida albicans Staphylococcus aureus* and *Escherichia coli* in different concentrations are shown in Figs. 3 and 4 There is no difference between MS and normals except for an increased response in the MS group to the highest candida concentration. Median responses and range are given in Table 2

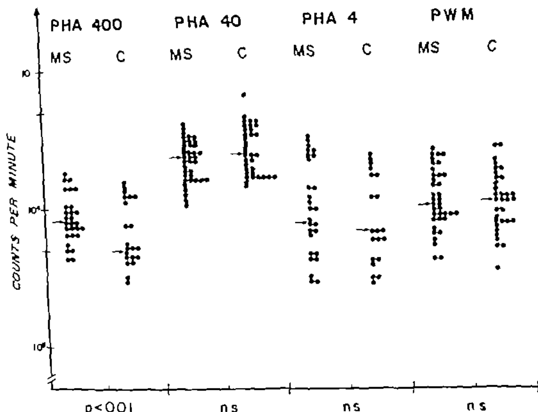


Fig 2 Counts per minute (increment) in lymphocyte transformation tests obtained by different concentrations of PHA and PWM, in MS patients and in normal individuals Arrow indicates median value.

TABLE 1 *Lymphocyte Transformation (Counts per Minute Increment) (cpm in Stimulated Cultures Minus cpm in Unstimulated Cultures) with Phytohaemagglutinin (PHA) and Pokeweed Mitogen (PWM) in Patients with Multiple Sclerosis (MS) and in Normal Individuals*

	MS			Control		
	Median	Range	Number	Median	Range	Number
PHA 400 μg per ml	8459	(2535–35846)	32	5120	(1392–15448)	27
PHA 40 μg per ml	23393	(7839–47792)	40	24543	(13984–64739)	37
PHA 10 μg per ml		not done		20419	(3818–35440)	27
PHA 4 μg per ml	7647	(824–30496)	34	6650	(1525–63559)	27
PWM 96 hours	10011	(1014–28472)	40	10690	(3452 26107)	37
PWM 120 hours		not done		12462	(5164–35928)	36

Only two patients showed more than *one* value below the range of values in the control group and both were normal with regard to all other parameters investigated for

Lymphocyte Subpopulations

The percentage of T cells in peripheral blood was slightly but significantly increased ($p < .05$) in the MS group (Fig 4) B cells were determined both by EAC-rosette for

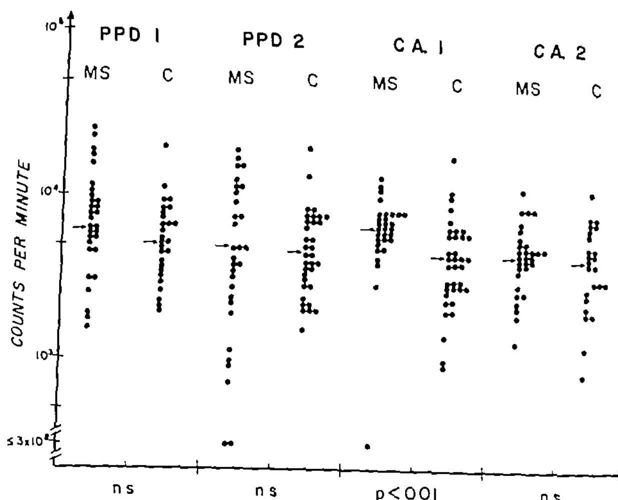


Fig 3 Counts per minute (increment) in lymphocyte transformation test obtained by two different concentrations of PPD and *Candida albicans* antigens in MS patients and normal individuals.

TABLE 2. *Lymphocyte Transformation (Counts per Minute Increment) with PPD (Tuberculin)* Candida albicans (C.A.) Staphylococcus aureus (S.A.) *and* Escherichia coli (E.Co.) *in Patients with Multiple Sclerosis and in Normal Individuals*

	MS			Control		
	Median	Range	Number	Median	Range	Number
PPD 1	6145	(–177–25451)	33	5100	(1984–20325)	27
PPD 2	4982	(41 19218)	31	4699	(914–20837)	36
C.A. 1	6632	(347 13706)	31	4587	(953–18686)	36
C.A 2	4553	(958–11995)	33	4389	(827–11780)	27
S.A. 1	4586	(–496–13681)	31	3872	(1569–10678)	36
S.A. 2	3618	(–581 10085)	29	3416	(803– 9479)	27
E.Co. 1		not done		2058	(296 12485)	36
E.Co. 2	2077	(–457– 9750)	33	2030	(12 9025)	27

mation and the presence of surface immunoglobulins (Ig⁺) Evaluated by the former method no difference between MS and controls could be found using the latter a slight, but significant ($p < .05$) increase in the per cent of Ig⁺ cells was found in the MS group

All p values given in the figures are those obtained by non-parametric rank sum test (Mann Whitney) If these p values were cor

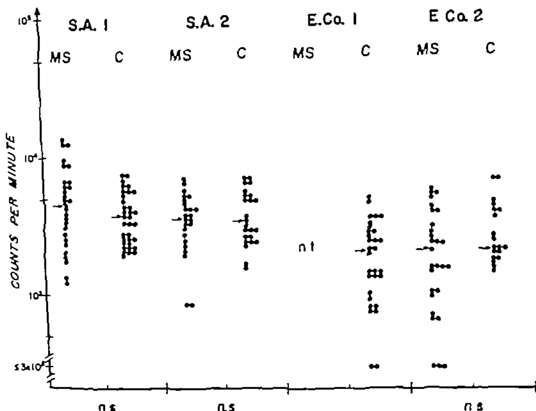


Fig 4 Counts per minute (increment) in lymphocyte transformation test obtained by two different concentrations of *Staph. aureus* and *E coli* antigens in MS patients and normal individuals.

TABLE 3 *Percentage of E-rosette Forming Cells (E-RFC) EAC-rosette Forming Cells (EAC-RFC) and Immunoglobulin Bearing Cells (Ig Cells) in Peripheral Blood from Patients with Multiple Sclerosis (MS) and from Normal Individuals*

		E-RFC	EAC-RFC	Ig⁺ cells
MS	Median (Range) Number	70 (55–85) 37	19 (9–36) 40	20 (10–33) 53
Control	Median (Range) Number	67.5 (52–80) 42	19 (8–26) 42	16 (8–29) 40

rected for by the numbers of comparisons (fourteen) none of the deviations between MS patients and those in the control group were found to remain significant.

DISCUSSION

Although it has been known for a long time that infections may provoke acute exacerbations of MS (26) it is a general clinical experience that MS patients neither are more susceptible to such infections, nor are they more defective in combating acute infectious diseases than normal individuals. Increased frequency of urinary tract infections in MS patients may be explained exclusively as lesions secondary to neurological disturbances of the bladder functions.

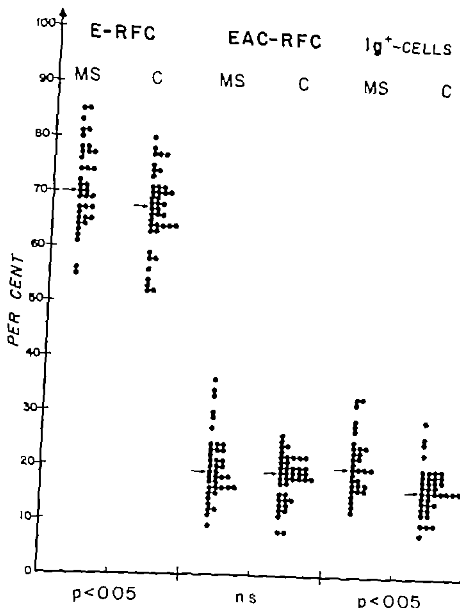


Fig 5 Percentage of T lymphocytes (E-RFC) and B lymphocytes (EAC-RFC and Ig cells) in MS patients and normal individuals.

This clinical experience is in agreement with the results presented in this report. The overall conclusion of these results is that the general immunological capacity of MS patients, as measured by these *in vitro* methods, does not differ from that of normal individuals.

The small differences observed in this study must be viewed in the light of the reproducibility of the test used and the number of comparisons made. The results of 39 repeated testings of two normal individuals on the same days showed a highly significant correlation (Fig 1) with the responsiveness to PHA of these 2 individuals when they were tested on different days. This indicates that there is a considerable day to-day variation of the test which is systematic because it affects all individuals tested simultaneously.

The material obtained from control individuals has been collected over an 18 months period for practical reasons, however larger groups of 5–10 MS patients have been tested per day. This will induce a day to-day variation which is smaller in the patient group than in the control group. Furthermore, the distribution according to age is not identical in the MS group and in the control group.

The change of finding significant deviations, i.e. $p < 05$ increases with increasing numbers of comparisons, and in this study we have made fourteen comparisons between the MS patients and the controls. Furthermore, only the increase in B cells has been confirmed by others (25 31)

Lisak and coworkers (25) found decreased numbers of T cells during acute exacerbations. All patients included in our study were in the stable phase of disease, and in this phase we did not find any definite abnormalities. No doubt, alterations in immunological reactivity will be encountered more often during the acute phase than during the stable phase of disease. As regards the acute phase, however it is difficult to decide whether these alterations are primarily involved or they are secondary phenomena. As MS is a disease which may run extremely variable courses (15) it is essential to examine comparable groups and the decrease in T cells during the acute exacerbation may be a phenomenon secondary to the disease itself.

Our results suggest that the general immunological capacity of MS patients is normal, but this does not exclude a possible involvement of the immunological system in the pathogenesis of MS. Firstly the parameters used are relatively rough and may fail to demonstrate minor changes in immune function. Secondly we are only investigating cells from peripheral blood, and phenomena such as trapping of active lymphocytes from the peripheral blood as well as difficulties in passing the blood brain barrier *must* be kept in mind. Thirdly although the results obtained by the leucocyte migration test (LMT) still are conflicting they may suggest a selective anergy towards the group of paramyxoviruses. Such a lacunar immune deficiency would be in agreement with animal models which have shown the existence of specific immune response (Ir) genes within the major histocompatibility system (MHS) in mice, rats and Rhesus monkeys (35) Although such Ir genes have not yet been *definitely* demonstrated in man, it is tempting to explain the previously mentioned association of the Dw2 determinant with the existence of an Ir gene or genes closely linked to this determinant, and responsible for an altered reactivity in MS patients.

The staff of the Municipal Hospital and the MS Rehabilitation Centre in Haslev is thanked for their kind cooperation, the skilled technical assistance from the technicians at the Tissue Typing Laboratory as well as the excellent secretarial assistance of *Elly Andersen* is greatly appreciated.

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REFERENCES

1 *Armitage P.: Statistical Methods in Medical* Research. Blackwell Sc. Publ., Oxford/London /Edinburgh/Melbourne, 1971

2. *Bertfeld H., Zaon, J A. & Donnenfeld, H.*. Cellular immunity in multiple sclerosis. Lancet i. 317 1976.

3 *Bratusch, Z., Douglas S D., Siegal, F P & Kunkel H G.* Human lymphocyte sheep erythrocyte rosette formation. Some characteristics of the interaction. Clin. Immun. & Immunpth. *1* 511–512, 1973.

4 *Bernsohn J & St phanides L. M* Aetiology of multiple sclerosis. Nature *215* 821–823, 1967

5. *Brody K. A., Harlem M M., Kurtcke, J F & White L. R.*. Unsuccessful attempt to induce transformation by cerebrospinal fluid in cultured lymphocytes from patients with multiple sclerosis. New Engl. J Med. *279* 202–204 1968.

6. *Brody J A. Sever J L. & Hensen, T E.* Virus antibody titers in multiple sclerosis Patients, siblings and controls. J amer med. Ass. *216*: 1441–1446 1971

7 *Broman, T., Bergmann L., F g, T., Gilland T Hyllested, K., Lindberg-Broman A M., P dersen, E. & Prasthus J.* Aspects on classification methods in multiple sclerosis. Proc. XVII Congr. Scand. Neurol. Acta Neurol. Scandinav *41* suppl. 13 543–548, 1965.

8 *Campbell, A M G Hersden, G Tatlow W F T & Whittle E. G.*. Lead in relation to disseminated sclerosis. Brain *73* 52–71 1950.

9. *Carp R. I., Lacuru, P C., Merz, P A & Merz, G S* Decreased percentage of polymorphonuclear neutrophils in mouse peripheral blood after inoculation with material from multiple sclerosis patients. J Exp. Med. *136*: 618–629 1972.

10. *Cendrowski W & Niedzielska K* Lymphocyte transformation induced *in vitro* by PHA and PPD in multiple sclerosis. J Neurol. Neurosur Psychiat 33 92–94 1970
11 *Ciongoli A K Platz P Dupont B Svejgaard A Fog, T & Jersild C* Lack of antigen response to myxoviruses in multiple sclerosis. Lancet ii 1147 1975
12. *Cunningham Rundels S Dupont B. Posner J B Hansen J A & Good R A* Lymphocyte transformation in vitro to paramyxovirus antigens in multiple sclerosis. Lancet ii 1204 1975
13 *Dau P C & Peterson R D A* Transformation of lymphocytes from patients with multiple sclerosis. Arch Neurol 23 32–40 1970
14 *Field E J & Caspary E A* Lymphocyte response depressive factor in multiple sclerosis. Brit. Med. J 4 529–532 1971
15 *Fog T & Linnemann F* The course of multiple sclerosis in 73 cases with computer designed curves. Acta Neurol. Scandinav 46 suppl. 47 1970
16 *Fuccilo D A Abena J E Traub R. C Gillespie M M Beadle E L. & Sever J L.* Cellular immunity in multiple sclerosis. Lancet i 980 1975
17 *Jensen M K* Lymphocyte transformation in multiple sclerosis. Acta Neurol Scandinav 44 200–206 1968
18. *Jersild C Dupont B Fog T Platz P & Svejgaard A* Histocompatibility determinants in multiple sclerosis. Transplant Rev 22 148–163 1975
19 *Jersild C Hansen G S Thomsen M Svejgaard A. & Dupont B.* Histocompatibility determinants in multiple sclerosis with special reference to the clinical course. Lancet ii 1221–1224 1973
20 *Knowles M Hughes D Caspary E. A & Field E. J* Lymphocyte transformation in multiple sclerosis. Inhibition of unstimulated thymidine uptake by a serum factor Lancet ii 1207–1208 1968.
21 *Knowles M & Saunders M* Lymphocyte stimulation with measles antigen in multiple sclerosis. Neurology 20 700–702 1970
22. *Koldovsky U Koldovsky J Henle G Henle W., Ackermann R & Haase G* Multiple sclerosis-associated agent Transmission to animals and some properties of the agent. Infect Immun. 12 1355–1366 1975
23 *Kouhscher L., Stenmit J & Ketelaer P* La croissance in vitro de leucocytes de malades atteints de sclérose en plaques. Acta neurol belg 66 274–288, 1966
24 *Link H* Oligoclonal immunoglobulin G in multiple sclerosis brains. J Neurol Sci. 16 103–114 1972.
25. *Lisak R P Levinson A I., Zweiman B. & Abdou N I* T and B lymphocytes in multiple sclerosis. Clin. exp. Immunol. 22 30–34 1975
26. *McAlpine D* Some aspects of the natural history In *McAlpine D Lumsden, C E. & Acheson E D* (Eds.) Multiple Sclerosis A Reappraisal. Churchill Livingstone, Edinburgh and London 1972, p. 100
27 *Mendes N F Tolnai M E A Silveira, N P A Gilbertsen R B. & Metzgar R. S* Technical aspects of the rosette tests used to detect human complement receptor (B) and sheep erythrocyte binding (T) lymphocytes. J Immunol. 111 860–867 1973
28. *Millar J H D Zilkha J K., Langman M J S Wright H P Smith A D Belin J & Thompson R H S* Double-blind trial of linoleate supplementum of the diet in multiple sclerosis. Brit. Med J 1 756–768, 1973
29 *Norrby E. & Vandvik B* Measles and multiple sclerosis. Proc. Roy Soc. Med. 67 1129 1132, 1974
30 *Nowak J & Walgt A* Surface markers on lymphocytes of multiple sclerosis patients Clin exp Immunol. 21 278–283 1975
31 *Oger J F Arnason B G W Wray S H & Kistler J P* A study of B and T cells in multiple sclerosis. Neurology 25 444–447 1975
32. *Platz P Dupont B. Fog T Ryder L. P Thomsen M., Svejgaard A & Jersild C* Mixed lymphocyte culture determinants, measles infection and multiple sclerosis. Proc. Roy Soc. Med. 67 1133–1136, 1974
33 *Salmi A Gollmar Y Norrby E. & Panelius M* Antibodies against three different structural components of measles virus in patients with multiple sclerosis, their siblings and matched controls. Acta path. microbiol. scand. Sect. B 81 627–634 1973
34 *Saunders M Knowles M & Field E J* Lymphocyte stimulation with phytohaemagglutinin in multiple sclerosis. Lancet i 674–675 1969
35 *Svejgard A Hauge M Jersild C Platz P Ryder L. P Nielsen L. Staub & Thomsen M* The HLA System. An Introductory Survey *Beckman I & Hauge M* (Eds) Monographs in Human Genetics 7 1976 S Karger Basel
36 *Utermohlen V & Zabriskie J B* Suppressed cellular immunity to measles antigen in multiple-sclerosis patients Lancet ii 1147–1148, 1973
37 *Utermohlen V & Zabriskie J B* A suppression of cellular immunity in patients with multiple sclerosis. J Exp Med 138 1591 1596 1973

Acta path. microbiol. scand. Sect. C, 84 511–519, 1976

LYMPHOCYTE TRANSFORMATION *IN VITRO* IN DERMATOPHYTOSIS

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Svejgaard, E., Thomsen, M., Morling, N. & Christiansen, Aa. Hein. Lymphocyte transformation in vitro in dermatophytosis. Acta path. microbiol. scand. Sect. C, 84 511–519 1976.

Peripheral blood lymphocytes from 59 patients with dermatophytosis and from nine young healthy women were studied by the lymphocyte transformation test (LT) using mitogens and bacterial as well as fungal antigens. The latter included *Candida albicans* (CA) and four dermatophyte species, i.e. *Trichophyton rubrum* (TR), *Trichophyton mentagrophytes* (TM), *Epidermophyton floccosum* (EF) and *Microsporum canis* (MF). Most of the patients showed normal transformation in response to mitogens and non-dermatophyte antigens, indicating that they have no functional T-cell deficiency. Dermatophyte antigens act as stimulators in LT. In general, patient lymphocytes responded more strongly to these antigens than lymphocytes from controls. In most patients suffering from TM infections, response to the TM antigen was significantly stronger ($p<0.05$) than that in the other patients, indicating that this antigen preparation shows species specificity. In patients with Trichophyton (TR + TM) infections, response to the corresponding antigens was significantly stronger than that in the other patients, which suggests the existence of genus specificity. Any differences between patients suffering from chronic TR infections and those with acute TR infections were not observed, a finding which is in contrast to those obtained in other studies. However, a few patients with chronic TM infections responded weakly to mitogens and non-dermatophyte antigens. LT in four patients with id-reaction to TM infection was not found to differ from that in the remaining TM patients.

Key words: Lymphocyte transformation; dermatophytosis; in vitro test.

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Dermatophytes form a modest group of botanically related fungi causing ring-worm infection (dermatophytosis) in man and animals. Three genera, viz. *Trichophyton*, *Epidermophyton* and *Microsporum* are known to exist; they comprise about 40 species, of which 23 are common human and veterinary pathogens. These fungi attack, proliferate and live in the horny layer of the epidermis, hairs and nails. Usually they do not invade skin beneath the horny layer or the internal organs. Dermatophytes occur all over the world and, although they are not harmful, they cause troublesome infections which may mean a problem to the public-health (5, 7).

The clinical findings in dermatophytoses are extremely variable, depending on the virulence and the origin of the dermatophyte, whether zoophilic or anthropophilic, as well as the response of the host (3, 7).

The primary manifestations include (1)

TABLE 1 *A Review of the Clinical Manifestations in the*

Clinical and mycological review of the patients							
Dermatophyte	♂	♀	Age, range, Average	Acute	Chronic	Id reaction	Kerion
Trichophyton rubrum	28	2	11–74 39	12	18	2	
Trichophyton mentagrophytes	11	1	21–79 42	5	7	6	
Epidermophyton floccosum	11	3	18–55 27	14			
Microsporum canis	1	1	12–24 18	2			
Trichophyton verrucosum		1	24	1			1
Total	51	8	11–79 30	34	25	8	1

acute i.e inflammatory lesions lasting for weeks or months, mostly located to the scalp glabrous skin (ring worm) inguinal folds, and feet, and (ii) *chronical* i.e eczematous, lesions lasting for years. The latter are mostly located on soles and/or palms, rarely generalized. In the course of the infection different skin reactions are noticed which can be explained as immunological reactions to the fungal antigens. The acute rash (id reaction) consisting of small vesicles on the hands and feet secondary to dermatophytosis of the feet, is frequent and simulates, histologically and clinically acute allergic contact dermatitis. Reactions of the antigen antibody complex type are seen as maculo-papular rashes, erythema multiforme and erythema nodosum (4) Finally urticaria to occur in the course of *T rubrum* infection has been described (12) Thus, the dermatophytes and their action in man offer several possibilities to study a variety of immunological reactions.

Until recently Intracutaneous testing with "Trichophytin®" was the only method by which hypersensitivity following dermatophytosis might be evaluated. A delayed type hypersensitivity following experimental dermatophyte infections is the usual type of immune reaction to be observed (5) Immediate type reaction may also occur but are mainly related to chronic infections and atopy (5 6) Precipitins and complement fixing antibodies can be detected in some cases of dermatophyte infections but are infrequent (5 11) *Balogh et al* (1) applied the lymphocyte transformation test (LT) to blood of eleven patients with dermatophytosis and candidosis and found positive stimulation in all cases. However normal controls were not included in their study

In this study 59 consecutive patients with dermatophytosis of various locations and caused by the common dermatophytes, were examined by the LT test to elucidate the cell mediated immunological response in acute, chronic, uncomplicated and complicated infections. Since this study was started, an independent study of the LT test applied to patients with *T mentagrophytes* and *T rubrum* infections has been reported by *Hanifin et al* (6)

PATIENTS AND METHODS

The series comprised 59 patients with dermatophytosis caused by *Trichophyton rubrum* (n = 30) *Trichophyton mentagrophytes* (n = 12) *Epidermophyton floccosum* (n = 14) *Microsporum canis* (n = 2) and *Trichophyton verrucosum* (n = 1) (Table 1) The majority of the infections were acute, while 18 patients with *T rubrum* and 7

...nts I volved in the Study and of the Isolated Dermatophytes

...n ringworm	Foot	Groin	Foot + groin	Groin + skin	Hand + foot	Generalized
	Location of dermatophytosis					
2	12	8	3		4	1
	11		1			
		12	1	1		
2						
1						
5	23	20	5	1	4	1

with *T. mentagrophytes* suffered from chronic infections. The controls were (i) nine young female technicians without clinical signs of dermatophytoses (group C_1). In addition (ii) control series of 38 individuals tested with mitogen and standard antigen stimulation has been incorporated (group C_2) (9).

Identification of the dermatophytes was done by microscopy (KOH 20 per cent) and culture of skin scales on Sabouraud dextrose agar containing penicillin, streptomycin and cycloheximid. The morphology of the cultures was determined by microscopy and, (10) if necessary further culturing was performed on Littman oxgall agar and cornmeal agar. The production of urease according to *Philpot* (8) was examined whenever the morphology of TR and TM was not characteristic.

The lymphocyte transformation test (LT) was performed in vitro as described elsewhere (9) using i) mitogens phytohaemagglutinin (PHA) and poke-weed mitogen (PWM) ii) antigens *Escherichia coli* (E. coli) *Staphylococcus aureus* (S. aur.) *Candida albicans* (C. alb.) and iii) dermatophyte antigens.

Dermatophyte antigens were prepared as water soluble extracts from each of four dermatophytes, i.e. *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Epidermophyton floccosum* and *Microsporum canis*. The details of this antigen preparation has been reported previously by *Christiansen & Svejgaard* (1976). Four-week-old cultures grown on Sabouraud dextrose broth 2 per cent were homogenized, lyophilized, resuspended in water and extracted in a Braun cell-homogenizer. After centrifugation, the protein content of the supernatant was measured according to Lowry. Finally the antigen extract was filtered through a Macropore® sterile filter and stored in small tubes at −80° C until use (2).

For the LT test, a stock-dilution containing 10 mg protein per ml antigen-dilution was made. Dilutions from 1:10 to 1:10,000 were prepared and, in some cases, dose-response curves were studied as shown in Fig. 1. For the final evaluation, the TR-antigen 1:1000 and the TM, EF, MC-antigens 1:100 were chosen because they induced strong responses in preliminary studies, and gave the best discrimination between patients and controls.

The stimulating capacity varied somewhat from batch to batch and, repeatedly, non-stimulatory batches were excluded. As far as possible, the same batch was used throughout, i.e. in the case of TR, one batch (batch 27) was used in 34 patients, in the case of TM, two batches (batch 28 and 33) were used in 36 patients, in the case of EF, three batches (batch 9, 12 and 22) were used in 45 patients, and in the case of MC, four batches (batch 8, 13, 19 and 30) were used in 49 patients.

In the LT method, 100,000 lymphocytes per culture tube were cultured in 0.5 ml RPMI-medium with 15 per cent pooled human serum from normal young, healthy, non-transfused male individuals, Hepes buffer and sodium bicarbonate. Cultures were set up with and without mitogen or antigen and incubated at 37° C in a humidified atmosphere with 5 per cent CO_2. Cultures with mitogens and antigens were routinely harvested after 96 and 120 hours of incubation, respectively. ¹⁴C-thymidin was added for the last 24 hours. Harvesting was done by a Skatron semi-automatic harvesting machine and the thymidine incorporation was measured in a liquid scintillation counter. The results are based

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	Location of dermatophytosis					
Sum [illegible]	Foot	Groin	Foot + groin	Groin + skin	Hand + foot	Generalized
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	11		1			
		12	1	1		
2						
1						
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Identification of the dermatophytes was done by microscopy (KOH 20 per cent) and culture of skin scales on Sabouraud dextrose agar containing penicillin, streptomycin and cycloheximid. The morphology of the cultures was determined by microscopy and, (10) if necessary further culturing was performed on Littman oxgall agar and cornmeal agar. *The production of urease according to Philpot (8)* was examined whenever the morphology of TR and TM was not characteristic.

The lymphocyte transformation test (LT) was performed in vitro as described elsewhere (9) using) mitogens phytohaemagglutinin (PHA) and poke-weed mitogen (PWM) ii) antigens *Escherichia coli* (E. coli) *Staphylococcus aureus* (S. aur.) *Candida albicans* (C. alb.) and iii) dermatophyte antigens.

Dermatophyte antigens were prepared as water soluble extracts from each of four dermatophytes, i.e. *Trichophyton mentagrophyte*, *Trichophyton rubrum*, *Epidermophyton floccosum* and *Microsporum canis*. The details of this antigen preparation has been reported previously by *Christiansen & Svejgaard* (1976). Four-week-old cultures grown on Sabouraud dextrose broth 2 *per cent* were homogenized, lyophilised, resuspended in water and extracted in [illegible] Brown cell-homogenizer. After centrifugation, the protein content of the supernatant was measured according to Lowry. Finally the antigen extract was filtered through a Microporer sterile filter and stored in small tubes at -80° C *until use (2)*.

For the LT-test, stock-dilution containing 10 mg protein per ml antigen-dilution was made. Dilutions from 1 10 to 1 10,000 were prepared and, in some cases, dose-response curves were studied as shown in Fig. 1. For the final evaluation, the TR-antigen 1 1000 and the TM EF MC-antigens 1 100 were chosen because they induced strong responses in preliminary studies, and gave the best discrimination between patients and controls.

The stimulating capacity varied somewhat from batch to batch and, repeatedly non-stimulatory batches were excluded. As far as possible, the same batch was used throughout, i.e. in the case of TR, one batch (batch 27) was used in 34 patients, in the case of TM, two batches (batch 28 and 33) were used in 36 patients, in the case of EF three batches (batch 9 12 and 22) were used in 45 patients, and in the case of MC, four batches (batch *8, 13 19 and 30)* were used in 49 patients.

In the LT method, 100,000 lymphocytes per culture tube were cultured *in 0.5 ml RPMI-medium* with 15 per cent *pooled human serum from normal* young, healthy non-transfused male individuals, *Hepes* buffer and sodium bicarbonate. Cultures were set up with and without mitogen or antigen and incubated *at 37° C in a* humidified atmosphere with 5 per cent CO_2. Cultures with mitogens and antigens were routinely harvested *after 96 and 120* hours of incubation, respectively. ^{14}C-thymidin was added for the last 24 hours. Harvesting was done by a 'Skatron' semi-automatic harvesting machine and the thymidine incorporation *was measured* in [illegible] liquid scintillation counter. The *results* are based

TABLE 1 *A Review of the Clinical Manifestations in the*

Clinical and mycological review of the patients							
Dermatophyte	♂	♀	Age, range, Average	Acute	Chronic	Id reaction	kerion
Trichophyton rubrum	28	2	11–74 39	12	18	2	
Trichophyton mentagrophytes	11	1	21–79 42	5	7	6	
Epidermophyton floccosum	11	3	18–55 27	14			
Microsporum canis	1	1	12–24 18	2			
Trichophyton verrucosum		1	24	1			1
Total	51	8	11–79 30	34	25	8	1

acute i.e inflammatory lesions lasting for weeks or months, mostly located to the scalp glabrous skin (ring worm) inguinal folds, and feet, and (ii) *chronical* i.e eczematous, lesions lasting for years. The latter are mostly located on soles and/or palms, rarely generalized. In the course of the infection different skin reactions are noticed, which can be explained as immunological reactions to the fungal antigens. The acute rash (id reaction) consisting of small vesicles on the hands and feet secondary to dermatophytosis of the feet, is frequent and simulates, histologically and clinically, acute allergic contact dermatitis. Reactions of the antigen-antibody complex type are seen as maculo-papular rashes, erythema multiforme and erythema nodosum (4) Finally urticaria to occur in the course of *T rubrum* infection has been described (12) Thus, the dermatophytes and their action in man offer several possibilities to study a variety of immunological reactions.

Until recently intracutaneous testing with "Trichophytin®" was the only method by which hypersensitivity following dermatophytosis might be evaluated. A delayed type-hypersensitivity following experimental dermatophyte infections is the usual type of immune reaction to be observed (5) Immediate type reaction may also occur but are mainly related to chronic infections and atopy (5 6) Precipitins and complement fixing antibodies can be detected in some cases of dermatophyte infections but are infrequent (5 11) *Balogh et al* (1) applied the lymphocyte transformation test (LT) to blood of eleven patients with dermatophytosis and candidosis and found positive stimulation in all cases. However normal controls were not included in their study

In this study 59 consecutive patients with dermatophytosis of various locations and caused by the common dermatophytes, were examined by the LT test to elucidate the cell mediated immunological response in acute, chronic, uncomplicated and complicated infections Since this study was started, an independent study of the LT test applied to patients with *T mentagrophytes* and *T rubrum* infections has been reported by *Hanifin et al* (6)

PATIENTS AND METHODS

The series comprised 59 patients with dermatophytosis caused by *Trichophyton rubrum* (n = 30) *Trichophyton mentagrophytes* (n = 12) *Epidermophyton floccosum* (n = 14) *Microsporum canis* (n = 2) and *Trichophyton verrucosum* (n = 1) (Table 1) The majority of the infections were acute, while 18 patients with *T rubrum* and 7

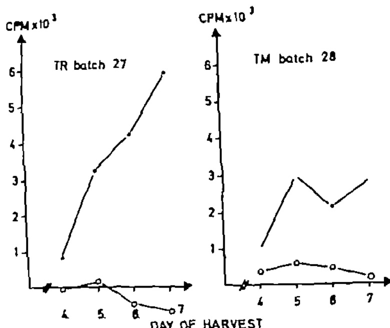


Fig 2 Time course of lymphocyte transformation. Increment counts per minute (cpm) of lymphocytes from one patient (●) and one control (○) stimulated by TR-antigen dilution 1 1000 and TM-antigen dilution 1 100 The lymphocytes were harvested at days 4 5, 6 and 7

No essential differences between the three groups are evident, and no statistically significant differences were found. Four patients show subnormal increment counts with PHA, and in one of these the PWM response was weak All four patients who had chronic athlete's foot caused by *T mentagrophytes* showed normal responses to the antigens. Another patient showed low counts to all three antigens, but normal PHA and PWM responses He suffered from pancytopenia and chronic dermatophytosis. Apart from this patient, only one in the entire series showed clinical diseases other than dermatophytosis. This patient suffered from systemic lupus erythematosus but showed normal LT responses.

The four different dermatophyte extracts were all capable of inducing lymphocyte proliferation, arbitrarily defined as increment counts per minute exceeding 500 (Fig. 4) In this figure, each control individual has been included only once. As regards individuals tested more than once, the average values are given. If all the separate control values were used in the statistical analysis, the significance would be even stronger than that to be obtained if each individual was included only once which, however was considered more correct. It appears from Fig 4 that lymphocytes in patients are stimulated to a higher degree than those in the controls. The differences were statistically significant for the TM and EF-antigens with P-values less than 0.01 and for the TR-antigen less than 0.05 while the difference between patients and controls stimulated with the MC-antigen was not found to be significant.

The specificity of the four different fungal extracts used as stimulators in the LT test was examined in the case of TR TM and EF-antigens, but not in the case of MC-antigen owing to the fact that the number of patients with MC-infection was small only In the six TM patients response to TM-antigen was strong in all cases, as compared with the greatly variable response in the remaining group this difference was significant (p less

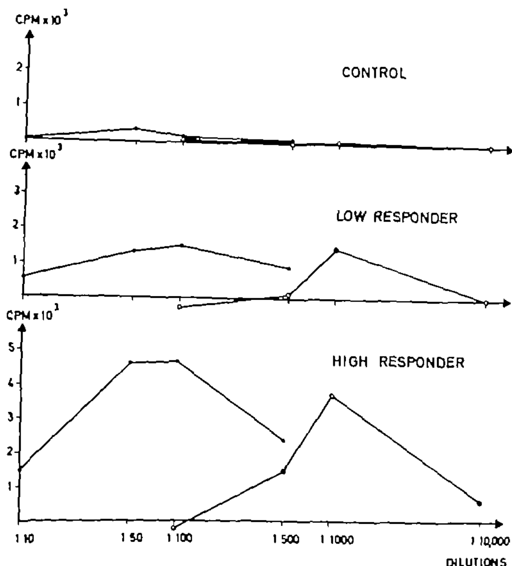


Fig 1 Dose-response curves of lymphocyte transformation. Increment counts per minute (cpm) of lymphocytes from one control and two patients (weak response and strong response) stimulated with TM (●) and TR (O) antigen in different dilutions.

on medians of triplicate culture and expressed as increment counts per minute (cpm) i.e. the difference between the cpm of the test and the cpm of the control culture without mitogen and antigen Statistical comparisons of groups were made by the Wilcoxon non-parametric rank sum test

RESULTS

Figs. 1 and 2 show representative dose response and time-course studies using TR and TM antigen, respectively It appears that these antigens give optimal stimulation in the dilutions 1 1000 and 1.50–1 100 respectively As to the kinetics of the responses to TR and TM of the antigen-dilutions used in the rest of the study it appears that there is a levelling off or even a decrease, in the increment counts per minute on day 6 followed by an increase on day 7 Similar observations were made in repeated experiments and to avoid possible irreproducibility on the days of late harvest we decided to harvest on day 5 Similar kinetic studies of EF and MS antigen formed the basis for the selection of the appropriate antigen concentrations to be used in the present study

The results of stimulation with the mitogens PHA and PWM and the antigens C alb. S aur and E. coli are shown in Fig 3 apply to all patients and the two control groups.

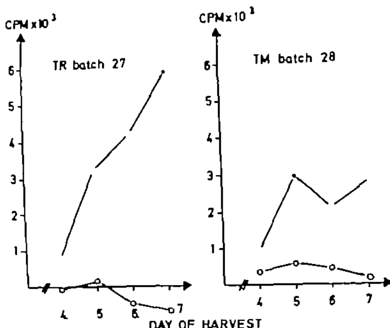


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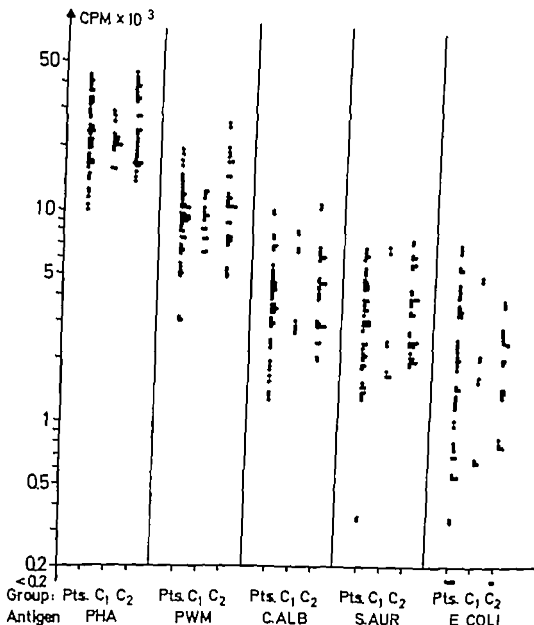


Fig 3 Lymphocyte transformation to mitogens and antigens. The increment counts per minute (cpm) of lymphocytes from patients (pts.) and control groups C_1 and C_2 stimulated with phytohaemagglutinin (PHA) Poke-weed mitogen (PWM) *Candida albicans* (C alb.) *Staphylococcus aureus* (S aur) and *Escherichia coli* (E. coli) antigen.

than 0 05) However this result must be evaluated with caution because of the small number of patients. No species specificity of the TR or EF antigens was observed

Considering the Trichophyton infected patients collectively (TM + TR) and comparing their responses to the TR and TM antigens, respectively in relation to responses in the remaining group of patients, some genus-specificity was suggested (Figs. 5 a and 5 b) For the TR antigen p was less than 0 05 and for the TM-antigen less than 0 01

Patients with chronic and acute *T rubrum* infections, respectively were compared (Fig 6) If stimulated with TR or with TM antigen, the reaction of lymphocytes in both groups would be almost indistinguishable

Four patients suffering from TM infection had id reaction. Comparison between these and the remaining patients showed no dif

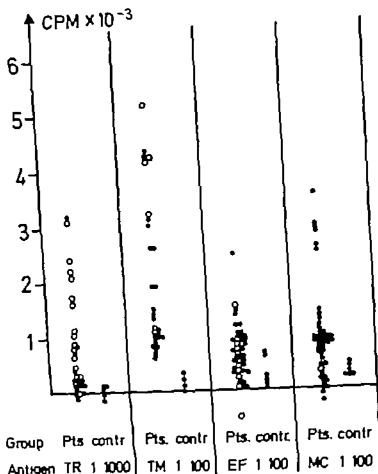


Fig 4 Lymphocyte transformation to dermatophyte extracts. Increment counts per minute (cpm) of lymphocytes from patients (pts.) and controls (contr.) stimulated with antigen extracts from *Trichophyton rubrum* (TR) batch 27 dilution 1 1000. *Trichophyton mentagrophytes* (TM) batches 28, 33 dilution 1 100 *Epidermophyton floccosum* (EF) batches 9 12, 22, dilution 1 100. *Microsporum canis* (MC) batches 8, 13 19 30, dilution 1 100. (o) indicates the patients suffering from a dermatophytosis corresponding to the antigen used. For TM and EF-antigens $p<0.01$ for TR antigen $p<0.05$, and for MC antigen n

ference in the degree of lymphocyte stimulation

DISCUSSION

The following conclusions can be drawn from the results. In general, lymphocytes in patients suffering from dermatophytosis gave normal responses to mitogens and antigens in the lymphocyte transformation test, thus indicating a normal T-cell function.

Extracts from dermatophytes can induce lymphocyte proliferation *in vitro* which confirms the observation reported by *Hanifin et al.* (6) who, however used another extraction procedure. The lymphocytes in patients with dermatophytosis were, on an average, stimulated by antigen extracts from four different dermatophytes to a higher degree than lymphocytes from normal control individuals. This difference is statistically significant for three out of four antigens and indicates that

Fig 5 a. Lymphocyte transformation to *T rubrum* antigen. Increment counts per minute (cpm) of lymphocytes from patients infected with TR or TM (TR + TM pts.) other patients and controls stimulated with TR antigen batch 27 (TR 27) dilution 1:1000 (O) = TM patients. For TR antigen $p < 0.05$

Fig 5 b Lymphocyte transformation to *T mentagrophytes* antigen Increment counts per minute (cpm) of lymphocytes from patients infected with TR or TM (TR + TM pts.) other patients and controls stimulated with TM antigen batch 28 plus 33 (TM 28 + 33) dilution 1:100 (O) = TM-patients For TM antigen $p < 0.01$

the patients are specifically sensitized to dermatophyte antigens. Comparative studies of the response to different dermatophyte extracts of lymphocytes in patients suffering from various dermatophytoses indicate the existence of genus and species specific lymphocyte stimulating antigens.

It is known from previous serological studies (2) that the antigen preparations used contain a great variety of different antigenic determinants on different molecules. It is possible that some of these factors may even have an unspecific mitogenic effect, while others may suppress lymphocyte transformation. Separation studies aiming at a clarification of these problems are in progress. Nevertheless, it is noteworthy that the two related fungi TR and TM appear to behave differently in dose response assays (Fig. 1) This observation was reproducible in all batches tested and is in accordance with the findings by *Hanifin et al.* (6) namely that TR antigens are active in higher dilutions than TM antigens.

In the independent study *Hanifin et al* (6) found that lymphocytes in persons suffering from chronic dermatophytosis caused by *T rubrum* did not respond to the specific

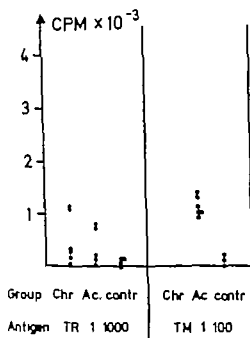


Fig 6 Lymphocyte transformation to *T rubrum* and *T mentagrophytes* antigen in acute and chronic dermatophytoses caused by *T rubrum* Increment counts per minute (cpm) of lymphocytes from patients with TR infections and controls stimulated with TR antigen, dilution 1 1000 and TM antigen, dilution 1 100 (Chr) = chronic. (Ac.) = acute.

antigen, thus indicating an inability to react with cell-mediated response to Trichophyton antigens. To elucidate this problem, patients with chronic TR infections were compared with those with acute TR infections (Fig 6) but no differences were found. Accordingly this study cannot confirm the hypothesis of a specific non-reactiv ty in patients with chronic TR infections as compared with patients w th acute infections. Nevertheless, some patients in both of these groups did not respond to TR antigen. The possibility that these low responses are clinically meaningful is subject to further study

Four patients w th id-reaction did not beha e significantly different from the re maining patients.

The expert technical assistance of Mrs. *Susanne Kahn*, Mrs. *Hanne A derson* Mrs. *Bodil Jakobse* Mrs. *Lillian M k* Mrs. *Anette Hoyer* Mrs. *Susanne Hertz* and Mrs. *A e-Marie Rulykke* is gratefully acknowledged. Furthermore w wish to thank *Arne Svejgaard* for helpful discussions during preparation of the manuscript.

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REFERENCES

1 *Balogh, E., Mészáros, C & Halmy K.* Die Anwendung des Lymphocytentransformationstestes bei der Untersuchung der mykotischen Sensibilisation. Mykosen *14* 207–211 1971
2. *Christiansen, A. H & Svejgaard E.* Studies on the antigenic structure of *Trichophyt n rubrum Trichophyton mentagrophytes, Epidermophyton floccosum* and *Microsporum anis* by crossed Immuno-electrophoresis. Acta path. microbiol. scand. Sect. C, *84* 337–341 1976.
3 *Emmons, C W Binfo d C H & Utz, J P.* Medical Mycology Sec. edition, Lea & Febiger Philadelphia 1970.
4 *F anks A G R senbaum E. M & Mandel, E. H Trichophyton ulf um* causing erythema nodosum and multiple kerion formation. Arch. Derm. Syph. 65 95–97 1952.
5 *G ppel, S F., Bishop C T & Blank F* Immunology of Dermatophytes and Dermatophytosis. Bact. Rev *38* ol. 2 222–250, 1974
6. *Hanifin J M., Ray L. E. & Lobitz, W C.* Immunolog.cal reacti ty in Dermatophytosis. Brit J Derm. *90* 1–8 1974
7 *Hildick-Smith G., Blank, H & Sarkany J.* Fungus diseases and their treatment. Churchill Ltd., London, 1964
8. *Philpot C* The differentiation of *Trichophyton mentagrophytes* from *T rubrum* by a simple test. Sabouraudia *5* 189–193 1967
9 *Platz P F g, T Morling, N., Svejgaard, A., Sonderstrup G Ryder L. P Thomsen M & Jersild C* Immunological in itro parameters in patients with multiple sclerosis and in normal individuals. Acta path. microbiol. scand. Sect. C, *84* 501–510, 1976.
10. *Rebell, G & T plin, D* Dermatophytes, their recognition and identification. University of Miami Press, 1970
11 *Svejgaard E. & Christiansen A. H.*, I preparation.
12. *Weary P E. & Guerrant J L.* Chronic urticaria in association with Dermatophytosis. Arch. Derm *95* 400–401 1967

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BRIEF REPORT

IMMUNOLOGICAL UNRESPONSIVENESS OF NUDE MICE TO LCM VIRUS INFECTION

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Christoffersen, P J., Volkert, M & Rygaard, J Immunological unresponsiveness of nude mice to LCM virus infection. Acta path. microbiol scand. Sect. C, 84 520–523 1976

The acute LCM infection in nude mice runs a course which is clinically inapparent, and neither a humoral nor a cell mediated immune response could be detected. Furthermore during this infection the blood virus titers reached extremely high values. These observations are contrary to what is seen when ordinary normal mice are infected with the LCM virus but identical with the events following infection of ordinary but immuno-deficient mice. Despite the absence of immune responses to the virus in the nude mice the very high virus titers obtained within the first few days of the infection declined somewhat during the following weeks. This and the other observations made are discussed briefly in the light of the current hypothesis concerning the immune status of the nude mice and the pathogenesis of the LCM virus infection in mice.

Key words Nude mice LCM virus immune reaction.

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The disease and death occurring in adult mice inoculated intracerebrally (i.c.) with the essentially non-cytopathogenic lymphocytic choriomeningitis (LCM) virus are dependent on an intact immune function (7 2) Recent exhaustive studies have revealed that the development of cell mediated immunity (CMI) parallels the elimination of virus after intraperitoneal inoculation, whereas the humoral response appears too late to be of any significance in the acute infection (6) Other workers have demonstrated that cytotoxic T lymphocytes can be obtained from the cerebro-spinal fluid of intracerebrally infected mice (13) Furthermore, the persistent virus carrier state which develops in neonatally infected mice can be abrogated only by the transfer of immune T lymphocytes (12) Strong evidence for the all important role of the T cells has therefore been obtained.

In many previous investigations concerning the effect of the immune response during LCM virus infection, immunosuppression by several means has been used extensively (irradiation, ALS treatment, cyclophosphamide etc.) The nude mouse which is claimed to be almost entirely deprived of a functioning T lymphocyte compartment (8) might also be immunologically non-reactive to the LCM virus, especially with regard to the cell-mediated lesions. This athymic mutant is now generally available and could be an additional useful and well-defined tool in future studies of the immunopathology of the LCM virus disease.

It is the aim of this brief report to draw a basic picture of the infection in nude mice. For this purpose the ordinary parameters—virus titer complement fixing (CF) antibodies and lethality—have been studied. As some workers have reported both strong and weak theta-positive lymphocytes from nude mice born of nu/nu-mothers (5) we have also found it necessary to perform an *in vitro* test for CMI

Materials and Methods

Mice. Nude mice of both sexes on C3H back ground, aged 4–6 weeks, reared under specified pathogen-free conditions, were obtained from Gl. Bomholtgård, Ry DK-8680, and were at least in the sixth backcross. They were kept under good conventional conditions in filter top cages and fed autoclaved pills and heat-treated drinking water *ad libitum*. To avoid flagellate infestation, metronidazol (3 g/l) was administered in the drinking water. The ordinary C3H mice were raised and bred in this laboratory. From this stock persistently infected mice (virus carriers) were produced by *intraperitoneal* inoculation of the newborns within 18 h after birth with 10^3 LD_{50} virus. Outbred Swiss mice were used for titration purposes.

Virus. The LCM virus strain was originally received from Dr. E. T[...]aub (Tubingen, Germany) and has been used in this laboratory for many years. All mice in these experiments were infected with 10^3 LD_{50} virus, harvested from the spleens of virus carriers. For infection of target L-cells in the cytotoxicity test, virus propagated in L-cells was used.

Virus titration. Serial 10-fold dilutions of tail vein blood were injected i.c. in groups of four outbred Swiss mice, and the titer was calculated by the method of *Kärber* (4) and expressed as $\log_{10}$ LD_{50}/0.03 ml intracerebral dose.

Complement fixation test (CF test). The standard procedure has been described in detail in previous paper from this laboratory (10). The method was modified only in the employment of Severs microtechnique (9). End points of the titrations were the last tubes showing less than 50 per cent haemolysis. The mice tested were killed and heartblood collected (puncture of the orbital plexus frequently elicited infection in the nude mice, and this procedure was therefore abandoned).

Cytotoxicity test. The test system used in this experiment is described in a previous paper (6). Briefly, suspensions of spleen lymphocytes were added to cultures of LCM virus-infected syngeneic ^{51}Cr labelled L-cells. The cytotoxicity of the spleen lymphocytes was measured by determining the amount of ^{51}Cr liberated after incubation for 18 hours. A cytotoxic index was calculated by the following equation:

$$\frac{^{51}Cr \text{ release in presence of immune lymphocytes} - {}^{51}Cr \text{ release in presence of normal lymphocytes}}{\text{Total } {}^{51}Cr \text{ incorporated} - {}^{51}Cr \text{ release in presence of normal lymphocytes}}$$

the test was performed using lymphocyte/L-cell ratios of 3.13:1, 6.25:1, 12.5:1 and 25:1.

Results

Mice inoculated i.c.: Seven nude mice were infected i.c. with 10^3 LD_{50} of virus. Neither clinical illness nor death occurred. Blood virus titers were determined on day 5, 10, 15, 30 and 46 in 3 to 4 mice and reached a maximum of 5.1 on day 30, as illustrated in Fig. 1. Thereafter the virus titer fell to 3.8. No CF antibodies were detected, even not on day 46.

Eight normal C3H mice which were also infected i.c., after eight days developed the typical tonic spasms and died.

Seven ordinary C3H virus carriers served as another control. The mean blood virus titer of these mice was 3.9.

Mice inoculated intraperitoneally (i.p.): Twelve nude mice were infected i.p., and the CF titers were measured in groups of three mice on day 10, 15, 20 and 30. No CF antibodies were detected. The rise of CF antibodies in the normal control mice is shown in Fig. 2. The blood virus titers stated in Fig. 3 were determined in groups of 3 to 6 nude mice, and the curve takes approximately the same course as that of the virus titers in i.c. infected nude mice. In the normal C3H control mice the wellknown transient viremia with a virus titer of 1.2 was seen.

CMI. In an earlier report the antiviral CMI in normal mice has been shown to reach maximum on day 9 (6). In the present study the degree of CMI was examined in 3 i.p. infected nude mice and in 3 normal i.p. infected C3H mice on day 10. The CMI was pronounced in normal mice as expressed by a cytotoxic index of 102 per cent in a lymphocyte/L cell ratio of 25:1. In nude mice significant cytotoxicity was not found by any ratio.

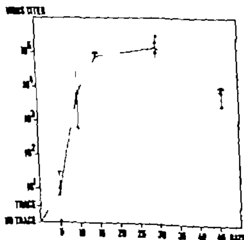


Fig. 1. Blood virus titers in nude mice following i.c. inoculation of 10^3 LD_{50} of virus. The graph is drawn through the mean titers. The single titers of each mouse are indicated by ○.

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strated in only 3 nude mice the fact that neonatally infected ordinary mice also show initially high virus titers and later on a decline (11) makes it very probable that the titers in the 3 mice are representative. The phenomenon of virus titer decline in the nude mouse as well as in the neonatally infected ordinary mouse is probably not an immunological event but rather a reflection of certain cellular factors influencing virus replication. In LCM virus-infected L-cell cultures, liberation of virus decreases and dies out if the cells are non-dividing (5). This might suggest that after a diffuse multiplication in most cells, virus replication ceases in non or slowly dividing cells, and that an equilibrium is established between virus production in dividing cells and virus decomposition.

The results of this study would suggest that the nude mouse is immunologically inert to the LCM virus challenge, and that therefore this mutant might be an interesting tool in further investigations of the problems concerning the immunopathological and immunohemopoietic disorders induced by the LCM virus.

The authors thank Dr *O. Marker* and Mrs. *G. Thörner Andersen* for performing the cytotoxicity test.

References: 1. *Burns W. H., Billups L. C. & Notkins A. L.*: Nature (Lond.) *256*: 654–656, 1975.– 2. *East J., Parrott D. V. & Seamer J.*: Virology *22*: 160–162, 1964.– 3. *Hotchin J.*: In *Lehmann-Grube F.* (Ed.): Lymphocytic choriomeningitis virus and other arena viruses. Springer Verlag, Berlin, Heidelberg, New York 1973, p. 85–99.– 4. *Körber G.*: Naunyn-Schmiedebergs Arch. Exptl. Pathol. Pharmakol. *162*: 481–483, 1931.– 5. *Lowe P., Hägg L., Mayer A. S. & Roelants G. E.*: Nature (Lond.) *255*: 656–657, 1975.– 6. *Marker O. & Volkert M.*: J. exp. Med. *137*: 1511–1525, 1973.– 7. *Rowe W.*: Proc. Soc. exp. Biol., N.Y. *92*: 194–198, 1956.– 8. *Rygaard J.*: Thymus & Self. Immunobiology of the mouse mutant nude. F.A.D.L., Copenhagen, and J. Wiley & Sons, London 1973.– 9. *Sever J. L.*: J. Immunol. *88*: 320, 1962.– 10. *Volkert M., Larsen J. H. & Pfau, C. J.*: Acta path. microbiol. scand. *61*: 268, 1964.– 11. *Volkert M. & Larsen J. H.*: Acta path. microbiol. scand. *63*: 161–171, 1965.– 12. *Volkert M., Bro-Jørgensen K., Marker O., Rubin B. & Trier L.*: Immunology *29*: 455–464, 1975.– 13. *Zinkernagel, R. M. & Doherty P. C.*: J. exp. Med. *138*: 1266–1269, 1973.

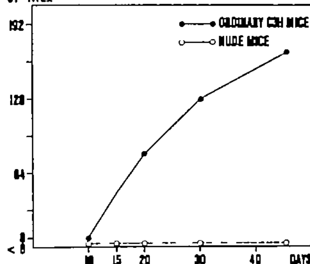


Fig 2 Complement fixing antibodies in nude mice and in ordinary C3H mice following i p inoculation of 10^3 LD_{50} of virus.

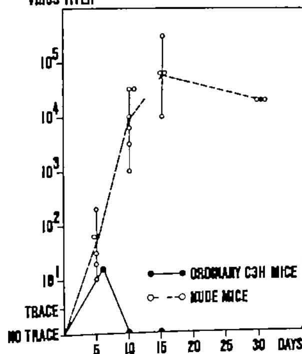


Fig 3 Blood virus titers in nude mice and in ordinary C3H mice following i.p inoculation of 10^3 LD_{50} of virus. The graph is drawn through the mean titers. The single titers of each nude are indicated by O

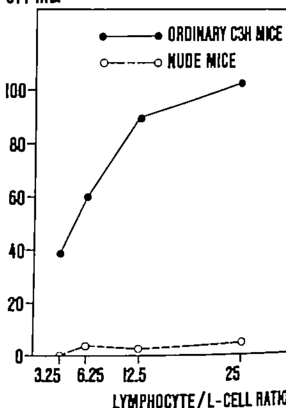


Fig 4 The cell mediated immune response to LCM virus in normal C3H mice and in nude mice 10 days after i.p. inoculation of 10^3 LD_{50} The magnitude of the response is expressed as the cytotoxic indices (CYT IND) of the lymphoid cells from the two groups of mice. Lymphocyte/L cell ratios tested 3 13 1 6.25 1 12.5 1 and 25 1

Discussion

Our results give no indication of any immune response in the LCM virus-infected nude mouse That no CMI to the virus could be detected is in full agreement with the assumption that the nude mouse is deprived of almost all the T lymphocytes. Moreover due to the fact that helper T cells are crucial for LCM antibody production (12) the absence of antibodies might also support this assumption. Using nude mice, *Burns et al.* (1) have shown this thymus dependency for antibody production to be a common feature for most viral antigens.

In the nude mouse the virus proliferated to high titers without causing any damage to the host, whereas in the normal mouse the same virus inoculum caused the death of the animal. In view of the immunological non-responsiveness to the LCM virus of the nude mouse this phenomenon lends further strong support to the 20-year-old hypothesis that it is the anti-viral immune response and not the virus itself that is responsible for the pathological damage.

The virus titer initially reached high values but on day 46 fell to the level of persistent virus carriers Even though the decline in titer was demon

strated in only 3 nude mice the fact that neonatally infected ordinary mice also show initially high virus titers and later on a decline (11) makes it very probable that the titers in the 3 mice are representative. The phenomenon of virus titer decline in the nude mouse as well as in the neonatally infected ordinary mouse is probably not an immunological event but rather a reflection of certain cellular factors influencing virus replication. In LCM virus-infected L-cell cultures, liberation of virus decreases and dies out if the cells are nondividing (3). This might suggest that after a diffuse multiplication in most cells, virus replication ceases in non or slowly dividing cells, and that an equilibrium is established between virus production in dividing cells and virus decomposition.

The results of this study would suggest that the nude mouse is immunologically inert to the LCM virus challenge, and that therefore this mutant might be an interesting tool in further investigations of the problems concerning the immunopathological and immunohemopoietic disorders induced by the LCM virus.

The authors thank Dr. *O. Marker* and Mrs. *G. Thörner Andersen* for performing the cytotoxicity test.

References. 1. *Barns, W. H., Billups, L. C. & Notkins, A. L.*: Nature (Lond.) *256*, 654–656, 1975.—2. *East, J., Parrott, D. V. & Seamer, J.*: Virology *22*, 160–162, 1964.—3. *Hotchin, J.*: In *Lehmann-Grube, F.* (Ed.): Lymphocytic choriomeningitis virus and other arenaviruses. Springer Verlag, Berlin, Heidelberg, New York 1973, p. 85–99.—4. *Kärber, G.*: Naunyn-Schmiedebergs Arch. Exptl. Pathol. Pharmakol. *162*, 481–483, 1931.—5. *Leor, F., Hägg, L., Mayor, K. S. & Roelants, G. E.*: Nature (Lond.) *255*, 656–657, 1975.—6. *Marker, O. & Volkert, M.*: J. exp. Med. *137*, 1511–1525, 1973.—7. *Rowe, W. P.*: Proc. Soc. exp. Biol., N.Y. *92*, 194–198, 1956.—8. *Rygaard, J.*: Thymus & Self. Immunobiology of the mouse mutant nude. F.A.D.L., Copenhagen, and J. Wiley & Sons, London 1973.—9. *Seiler, J. L.*: J. Immunol. *88*, 320, 1962.—10. *Volkert, M., Larsen, J. H. & Pfau, C. J.*: Acta path. microbiol. scand. *61*: 268, 1964.—11. *Volkert, M. & Larsen, J. H.*: Acta path. microbiol. scand. *63*, 161–171, 1965.—12. *Volkert, M., Bro-Jørgensen, K., Marker, O., Rubin, B. & Trier, L.*: Immunology *29*, 455–464, 1975.—13. *Zinkernagel, R. M. & Doherty, P. C.*: J. exp. Med. *138*, 1266–1269, 1973.

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